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In Vitro S-Glutathionylation of S-Nitrosoglutathione Reductase from Arabidopsis Thaliana and Phenotype Determination of Sensitive to Formaldehyde 1 Knockout Strains of Saccharomyces Cerevisiae

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IN VITRO S-GLUTATHIONYLATION OF S-NITROSOGLUTATHIONE REDUCTASE FROM *ARABIDOPSIS THALIANA* AND PHENOTYPE DETERMINATION OF *SENSITIVE TO FORMALDEHYDE 1* KNOCKOUT STRAINS OF *SACCHAROMYCES CEREVISIAE*

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By

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ABSTRACT

I*N VITRO* S-GLUTATHIONYLATION OF S-NITROSOGLUTATHIONE REDUCTASE FROM *ARABIDOPSIS THALIANA* AND PHENOTYPE DETERMINATION OF *SENSITIVE TO FORMALDEHYDE 1* KNOCKOUT STRAINS OF *SACCHAROMYCES CEREVISIAE*

FEBRUARY 2018

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Cells are constantly exposed to different stresses – one being redox stress, which is induced by metal, reactive oxygen species and reactive nitrogen species. S-nitrosoglutathione reductase (GSNOR) helps modulate redox stress by two different mechanisms – either by reducing S-nitrosoglutathione (GSNO) to oxidized glutathione (GSSG) or by oxidizing hydroxymethyl glutathione (HMGSH), a biproduct of glutathione and formaldehyde, to formic acid. GSNO has the potential to posttranslational modify proteins in two different manners, either by S-nitrosation or by S-glutathionylation. Interestingly, GSNOR can be modified by its substrate GSNO, either by S-nitrosation, which has previously been reported, or, as discussed in this thesis, by S-glutathionylation. As S-glutathionylation has been reported to occur through intermediate species, the S-glutathionylation of GSNOR appears to occur though the S-nitrosated intermediate, instead of the most common route of an oxidation pathway. It is hypothesized that the S-glutathionylation, and the overall presence of glutathione, can act as a buffer to regulate the amount of nitrosation that GSNOR experiences, and thus the enzymatic activity. It is has reported that the S-nitrosation occurs on three different non-structural, non-catalytic, solvent-accessible cysteine residues. Experimentation was conducted using *Saccharomyces cerevisiae* as

a model organism to determine how those three cysteine residues of the GSNOR homolog Sensitive to Formaldehyde 1 (*SFA1*) participate in the indirect detoxification of formaldehyde, through the hydroxymethyl glutathione pathway. It has been determined that cysteine 370 is not as important as previously thought, but the other one or two cysteines (either cysteine 10 or 271) do indeed play a role in the detoxification, but further analysis needs to be conducted.

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CHAPTER 1
BACKGROUND

a. Introduction

Throughout the course of an organism's lifetime, it is exposed to a multitude of different stressors. One type of stress is redox stress, which occurs from exposure to reactive metals, oxidative and/or nitrosative agents (Sies 1997; Jomova and Valko, 2011) A way to modulate the impact a redox stressor has on the organism and its proteins, is by utilizing post-translational modifications of proteins to their over-oxidation and/or regulate their activity, structure, and function (Mieyal and Chock, 2012; Grek et al, 2013). This thesis primarily focuses on the *in vitro* post-translational cysteine modification on the enzyme S-nitrosoglutathione reductase (GSNOR) during redox stress. Additionally, work was performed in an *in vivo* system to decipher the role of three highly conserved non-structural, non-catalytic, solvent-accessible cysteines and their involvement in regulation of GSNOR during cellular stress.

b. *Arabidopsis thaliana*

Arabidopsis thaliana is one of the most commonly used model organisms for studies of plant biology. *A. thaliana* possesses multiple attributes that allow it to be such an optimal and beneficial organism to use for studying plant biology; some beneficial attributes include, but are not limited to, small size, a rapid life cycle, being a diploid organism, simple protocols for genetic transformation, and having a fully sequenced genome. The entire 125 Mb genome of *A. thaliana* was fully sequenced by the year 2000, and there are many reference materials, such as libraries of specific genetic knockouts, that allow for detailed exploration of plant biology and corresponding genetic pathways (Arabidopsis Genome Initiative, 2000; Weems et al, 2004; Baxevanis, 2006).

While most of this thesis involves *in vitro* experiments related to regulation of GSNOR, the motivation for studying this enzyme came originally from identifying a GSNOR mutant in *A. thaliana*. Plants lacking GSNOR activity have a number of severe phenotypes, including reduced root branching, increased shoot branching, and highly reduced fertility. The goal of this thesis is insight into the regulation of GSNOR from *in vitro* studies of the protein from *A. thaliana* in order to better develop hypotheses about how this enzyme *in vivo*. While *in vitro* studies comprise a highly simplified system, they are useful for basic exploration of the regulation and mechanisms of specific enzymes.

c. *Saccharomyces cerevisiae*

Saccharomyces cerevisiae, otherwise known as Baker's yeast, is a prevalent fungal model organism used in studies of gene expression and cell biology. *S. cerevisiae* has a doubling time of approximately 1.5 hours when grown at its optimal temperature of 30°C. It can be grown in either haploid or diploid phases, which allows for easy construction of gene knockout stains to study the direct relationship between genes and phenotype. The growth cycles of *S. cerevisiae* in culture consists of three phases: lag, exponential, and stationary phase (Fig. 1). Each phase has a slightly altered metabolic balance of glycolysis and respiration, with the exponential phase being the most commonly used when conducting experiments with *S. cerevisiae* cells, as that is when the Kreb's cycle of glucose respiration is taking place (Frick and Whittmann, 2005; Bento et al, 2016).

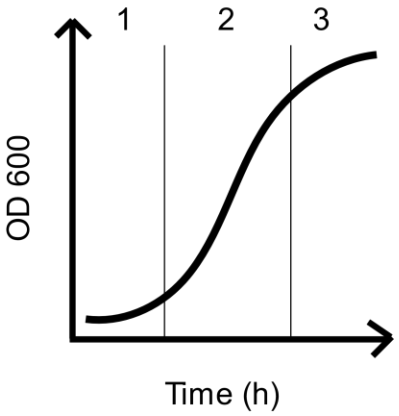
While most of this thesis focuses on *in vitro* experimentation with purified *A. thaliana* GSNOR, multiple *in vivo* experiments were conducted in *S. cerevisiae* with the goal of understanding how GSNOR operates *in vivo*. *S. cerevisiae* was used as a model organism for *in vivo* experiments rather than *A. thaliana* because of its rapid growth and the ability to simply and rapidly perform

genetic manipulations through homologous recombination or introduction of autonomously replicating plasmids. This allows faster analysis of genetic alterations in comparison to plants.

These attributes of *S. cerevisiae* make it a suitable model organism for determining how a specific enzyme, in this case, which in yeast has been named SENSITIVE TO FORMALDEHYDE 1 (SFA1), operates in a eukaryotic cell. While it is not a perfect substitute for studying the biological processes in *A. thaliana*, experiments should provide insight into the enzymatic regulation and cellular processes in which GSNOR/SFA1 is engaged.

Figure 1. Typical growth phase of *S. cerevisiae* cells in batch cultures

A schematic of a standard growth curve for a culture of *S. cerevisiae* cells. There is an initial slow growth phase – lag phase (1). Then the cells reach an exponential growth phase (2) where normal glucose-mediated cellular respiration occurs, and cells are typically harvested for experimentation. When nutrients become depleted, the cells reach stationary phase (3), where they maintain the same OD and undergo mitochondrial respiration and cell death.



1) Lag phase
2) Exponential phase
3) Stationary phase

d. Stressors and modulating redox stress though post-translational modifications (PTMs)

Throughout an organism's lifecycle, can be exposed to a multitude of different stressors – environmental, chemical, abiotic, and others. One aspect of adaptation to stress involves post-translational modifications (PTMs) of proteins. PTMs can result in the alteration of an enzyme's activity, structure, and/or localization to respond to new cellular demands (Deribe, 2010; Duan and Walther, 2015). While there are many different stressors and PTMs, such as phosphorylation, ubiquitination and prenylation, this thesis focuses on redox stressors and redox-related PTMs.

Common forms of redox stressors include, but are not limited to, exposure to metals and oxidative, and/or nitrosative agents (Sies 1997; Giles et al, 2003; Jomova and Valko, 2011). These stressors can induce PTMs, such as reversible or irreversible oxidation of proteins or S-nitrosation of protein thiols (Hess, 2005). Reversible oxidation of thiols to sulfenic acid (SOH) can have substantial effects on a protein's function. Sulfinic acid (SO_2H) oxidation of thiols tends to be biologically detrimental, but it can be enzymatically removed by sulfiredoxins. When protein thiols are fully oxidized to sulfonic acid (SO_3H), the modification is irreversible biologically and typically eliminates protein function (Cai and Yan, 2013). These thiol modifications can have regulatory downstream effects, altering cellular function. (Deribe, 2010). There are at least three ways to these modifications caused by redox stressors can be regulated: buffering cellular redox potentials, blocking the accessibility of the reactive thiol group, and through enzymatic regulation, such as sulfiredoxins (Finkel, 2000; Aquilano et al, 2014)

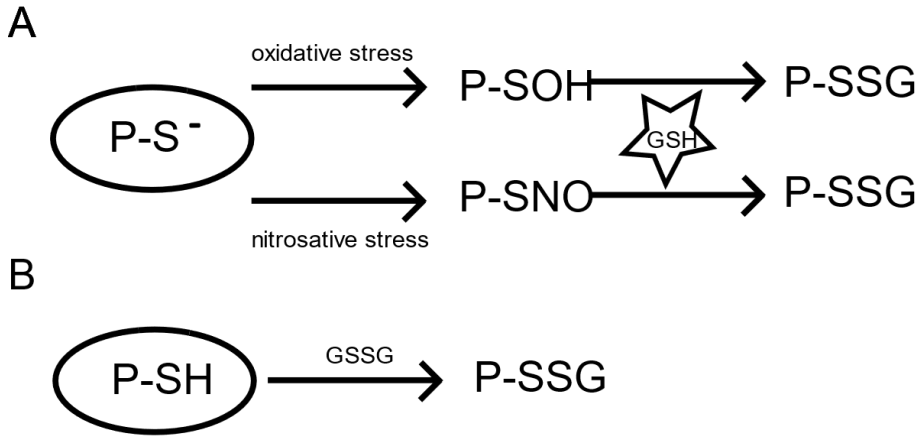
The most abundant cellular redox buffer is the tri-peptide glutathione (GSH), which can either exist in a reduced (GSH) or an oxidized state (GSSG). In a normal, unstressed cellular environment the ratio of GSH:GSSG tends to be about 100:1 (Zitka et al,2012). However, during

oxidative stress this ratio is significantly altered and can get shifted as low as 4:1 (Aquilano et al, 2014). The ratio of reduced-GSH to oxidized GSSG is constantly changing with the environment of the cell. GSH can interact with oxidative agents as a buffer, which in turn creates GSSG, which is reduced back to GSH by glutathione reductase (Couto, 2016). GSH can react with reactive nitrogen species, such as nitric oxide or peroxynitrite, to form S-nitrosoglutathione (GSNO), which can then transfer the nitroso group to free reactive thiols on proteins (Finley et al, 1981; Broniowska et al, 2013).

Another way in which redox stressors can be prevented from oxidizing proteins, is for the protein thiols to first be blocked to prevent their modification with other reactive species. It has been suggested that S-glutathionylation, the addition of GSH to a reactive thiol, is an *in vivo* mechanism to protect against irreversible protein over oxidation (Mieyal and Chock, 2012; Barinova et al, 2017). There is more than one way in which a protein thiol can undergo S-glutathionylation (Fig. 2). However, it has been noted that specific proteins are glutathionylated by specific pathways involving defined intermediate species (Grek et al, 2013). Glutathionylation has diverse effects on proteins. Some proteins exhibit an increase in activity, such as human cystathionine β-synthase and interleukin-1β, upon glutathionylation, while other proteins show a decrease in activity, such as GAPDH and eNOS (Mohr et al, 1999; Chen et al, 2010; Niu et al, 2015; Zhang et al, 2017) This thesis explores the occurrence of S-glutathionylation on *A. thaliana* GSNOR during redox stress.

Figure 2. Potential pathways of protein S-glutathionylation

Potential pathways of protein S-glutathionylation. (A) The free thiol group can be subjected to oxidative or nitrosative agents, causing either single oxidation (-OH) or nitrosation (-NO), which can in turn be modified by GSH, resulting in S-glutathionylation. (B) The thiol group can be activated at a physiological pH to a thiolate anion (S^-), which can then act as the nucleophile to attack oxidized glutathione (GSSG), resulting in S-glutathionylation.



e. S-nitrosoglutathione reductase (GSNOR) and previous work

S-nitrosoglutathione reductase (GSNOR), is a class III alcohol dehydrogenase also known as formaldehyde dehydrogenase (FALDH). It is a cytosolic enzyme that regulates the main intercellular reservoir of nitric oxide, S-nitrosoglutathione (GSNO) (Liu et al, 2001; Xu et al, 2013). GSNOR was originally identified as an enzyme that has NAD^+-dependent formaldehyde dehydrogenase activity (Fig. 3B) in pea seeds, as it decomposes hydroxymethyl glutathione (HMGSH), an intermediate in formaldehyde detoxification. However, it was later confirmed that it is involved in regulation and decomposition of GSNO in a NADH-dependent manner (Uotila and Koivusalo, 1979; Jensen et al, 1994; Liu et al, 2001). As seen in Figure 3A, GSNOR acts upon GSNO by irreversibly decomposing GSNO in a NADH-dependent manner to ammonia (NH_3) and reduced glutathione (GSH), which in turn gets oxidized to GSSG. By irreversibly reducing GSNO, GSNOR regulates the amount of cellular reactive nitric oxide species and thereby indirectly regulates the amount of protein S-nitrosation (Brzezek, 2014). Disrupting the activity of GSNOR *in vivo*, leads to defects in the development of lymphocytes, complications in neural development, and neuromuscular atrophy (Yang et al, 2010; Montagna et al, 2014; Barnett, 2017).

The active form of GSNOR from *A. thaliana* (AtGSNOR) is a homodimer of two 40689 Da monomers, of 379 amino acids (Fig. 5). Interestingly, each monomer of AtGSNOR contains 15 cysteine residues, which gives AtGSNOR a mole percent cysteine of 3.84% compared to the mole percent cysteine of 1.37% of all total proteins in the UniProtKB Database as of 2013 (Xu et al, 2013). Cysteines tend to be evolutionary conserved as they are often critical residues for protein function and stability (Giles et al, 2003). As seen in Figure 4, the overall amino acid conservation between AtGSNOR and the *Homo sapiens* and *Saccharomyces cerevisiae*

homologs is 68.45% and 60.16% respectively, with many of the cysteine residues conserved between those three organisms. The cysteine residues in GSNOR have either a structural, catalytic, or unknown function. Each monomer of GSNOR from *A. thaliana* binds two zinc 2^+ ions, with four cysteine residues (C99, C102, C105, and C113) that bind a structural zinc ion, while the active site zinc is bound by two catalytic cysteine residues (C47 and C177), a histidine residue, and a water molecule. However, there are three cysteines in AtGSNOR (C10, C271, and C370), that are conserved across the plant kingdom, and are highly conserved between the human and *S. cerevisiae* homologs that are highlighted in Figure 4. Those three conserved cysteines are non-structural, non-catalytic, solvent-accessible cysteine residues, which suggests that they hold some other specific importance. The positioning of those three cysteines can be seen in Figure 5. C10 and C370 are on the outer region of the protein, while C271 is closer to the active site and dimer interface. It has been reported that those three cysteine residues can be S-nitrosated *in vitro* by the nitric oxide donors GSNO, CysNO and SNP (Guerra et al, 2016). The resulting protein S-nitrosation decreases the enzymatic activity of GSNOR (Guerra et al, 2016). It was reported that S-nitrosation seems to be primarily occurring on C370 but can occur on all three specific cysteine residues *in vitro*. Since S-nitrosation can occur on these cysteine residues, there is potential that other redox related post-translational modifications, specifically S-glutathionylation, can occur on these same residues as well.

Figure 3. Activity of GSNOR

GSNOR has two activities. A) GSNOR irreversibly reduces GSNO to final products of ammonia (NH_3) and GSSG in a NADH-dependent manner. B) GSNOR oxidizes HMGSH to formic acid (HCOOH) and GSH in a NAD^+-dependent manner.

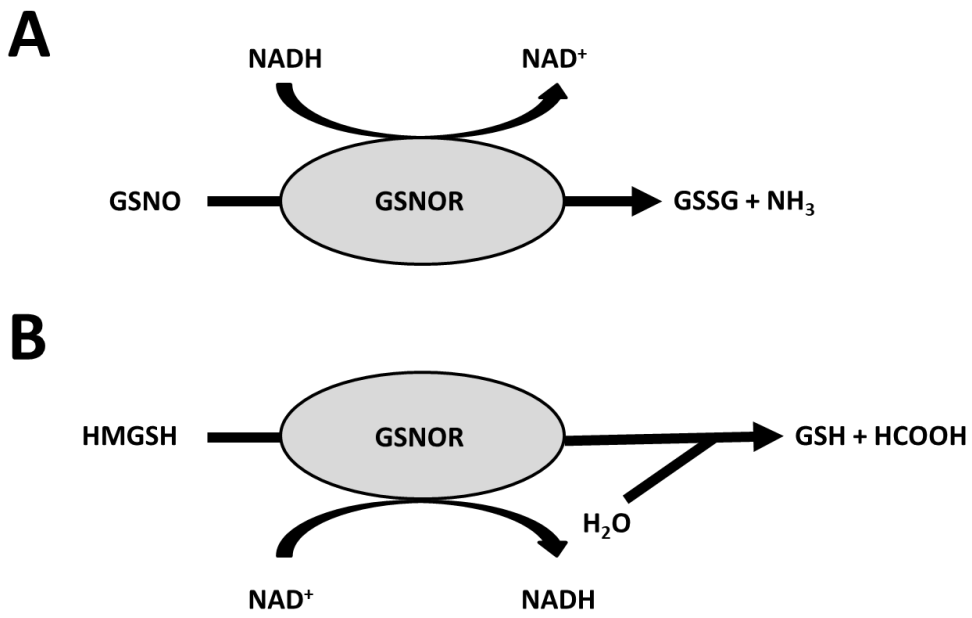


Figure 4. Amino acid alignment of the GSNOR orthologs of *A. thaliana, H. sapiens,* and *S. cerevisiae*

An amino acid alignment was performed using the Clustal Omega multiple alignment software (version 1.2.4, 2017) for the GSNOR orthologs of *A. thaliana* (Q96533)**,** *H. sapiens* (P11766) and *S. cerevisiae* (P32771). The "*" denotes exact amino acid conservation, while ":" denotes similar amino acids. The cysteines have been marked in color; red corresponding to zinc binding cysteines, blue to the non-catalytic, non-structural, solvent-accessible cysteines of interest, and green to other cysteines in the protein. Overall percentages of amino acid conservation are listed in the lower-right corner.

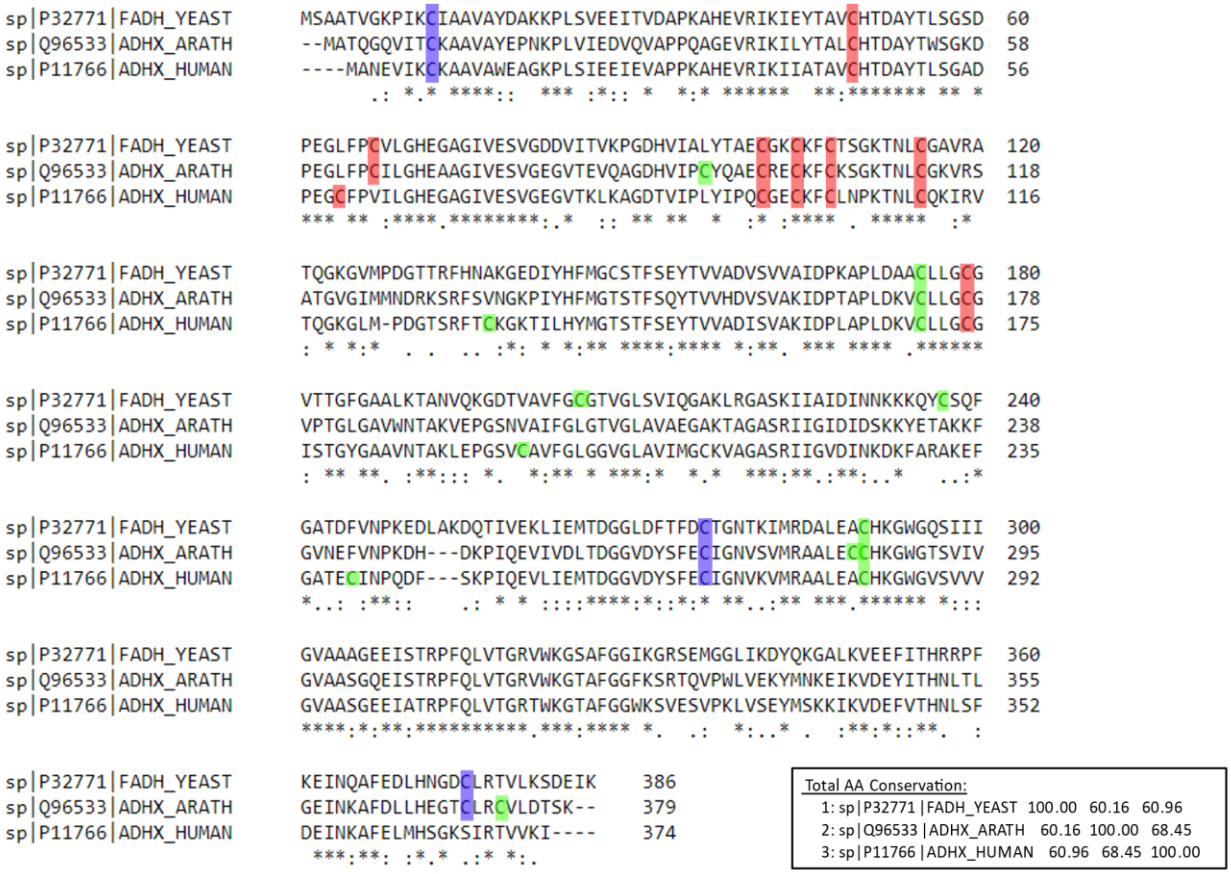
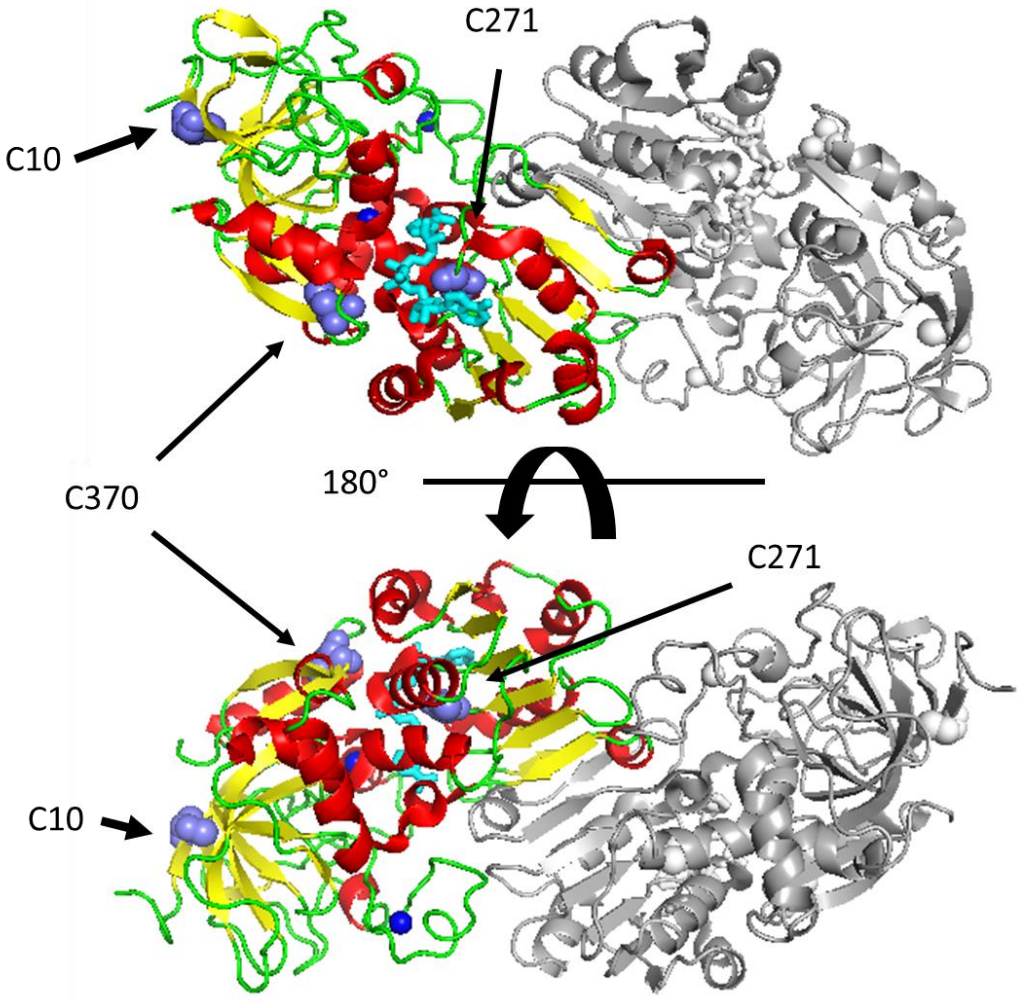


Figure 5. Structure of AtGSNOR

Representative model of the AtGSNOR dimer using PDB file 4JJI. One monomer is in greyscale, while the other is in color to highlight the secondary structure features. Alpha helices are red, beta sheets are yellow, and unstructured regions are green. The three cysteines, C10, C271, and C370, are shown in purple. The two zinc ions are dark blue spheres, and the NAD^+ molecule is in cyan. C271 is the closest of the three cysteines to the active site and the dimer interface.



f. Thesis overview

Whether GSNOR is redox-redox regulated and the potential mechanism of regulation is unclear. GSNO, the primary substrate of GSNOR, can either S-nitrosate or S-glutathionylate reactive cysteine residues. GSNOR is a cysteine rich protein, with three specific cysteine residues that are highly conserved, non-structural, non-catalytic, and solvent-accessible. It has been previously determined that those three cysteine residues can be S-nitrosated (Guerra et al, 2016). In this thesis I set out to determine whether GSNOR can be S-glutathionylated *in vitro* under redox stress conditions, and if S-glutathionylation has a role in maintaining enzymatic activity. I will also address how those three conserved cysteines play a role in an *in vivo* system by using the model organism *S. cerevisiae* and GSNOR cysteine to alanine mutants to compare phenotypic differences after stress.

Chapter 2 describes the methods and conditions used in all experiments. Chapter 3 explores *in vitro* S-glutathionylation of GSNOR, residue specificity of S-glutathionylation, and the impact of this modification on enzymatic activity. Chapter 4 seeks to elucidate phenotypic differences between wildtype and sfa1Δ *S. cerevisiae* cells, and whether conserved cysteine residues of SFA1/ AtGSNOR demonstrate a role in the stress response phenotype. Chapter 5 will outline future work for this project.

CHAPTER 2
METHODS

a. Chemicals and reagents

All reagents were purchased from Sigma-Aldrich unless otherwise stated. S-nitrosoglutathione (GSNO) was made in-house using the method described by Duong et al.(2013) and stored at 100 mM in 200 µl aliquots at -80°C and thawed on ice as needed. 100 mM S-nitrosocysteine (CysNO) was prepared using the method described by Kumar et al, 2013 and used the same day.

b. Proteins and mutants

GSNOR from *Arabidopsis thaliana* (AtGSNOR, AT5G43940.1) was expressed as a N-terminal polyhistidine fusion protein, either with or without an N-terminal His-SUMO-tag in pET expression plasmids (Novagen). The *AtGSNOR* gene was amplified, cut, and ligated as described in Guerra et al (2016). The AtGSNOR cysteine 271 to alanine (AtGSNOR C271A) mutant was created by using the Stratagene quick-change method (Agilent) and the substitution was confirmed by DNA-sequencing as well as mass spectrometry of the purified protein.

c. Protein purification

Wild-type AtGSNOR and the C271A mutant were transformed into BL-21 pLyss *E. coli* cells and grown to an OD_{600} of 0.4-0.6, treated with a final concentration of 0.5 mM IPTG and incubated at 16°C overnight. Cells were collected by centrifugation at 10,000 x *g* at 4°C for 10 min. Cells were resuspended in 20 ml lysis buffer ([50 mM NaH_2PO_4 + 200 mM NaCl + 10 mM Imidazole pH 8.0] + 0.5x EDTA-free Complete Protease Inhibitors (Roche)) per 1 L of cells. The cells were then subjected to sonication and processed through a microfluidizer to lyse the cells. The slurry was then incubated with 1 ml of lysis-buffer washed nickel beads at 4°C for 1 hr. The

beads were subsequently washed twice and then eluted with [50 mM NaH_2PO_4 + 300 mM NaCl + 250 mM Imidazole pH = 8.0] in a separate tube. Protein concentration was determined by absorbance at 280 nm using the extinction coefficient of 42400 L mol^{-1} cm^{-1} for wildtype AtGSNOR and C271A. Purity of the eluted protein was analyzed by SDS-PAGE (12% acrylamide) and Coomassie Blue R-250 protein stain. Purified protein was distributed into 200 µl aliquots of 80 µM and 45 µM for AtGSNOR and C271A respectively and stored at -80°C. Protein was thawed on ice as needed and refrozen up to two times per aliquot.

d. *In vitro* modification of GSNOR

GSNOR was treated with multiple different reagents to induce PTMs. For all reactions, 20 µM of GSNOR was initially treated with 0.3 mM dithiothreitol (DTT) for 30 min in 50 mM potassium phosphate pH=7.2 to fully reduce the protein. For the GSNO treatments, reduced GSNOR was treated with 2 mM GSNO for 1.0 hr at room temperature in the dark. For the altered GSSG treatment, GSNOR was treated with 4 mM GSSG for 1.0 hr at room temperature in the dark. For the oxidative stressor treatments, reduced GSNOR was treated with 0.5 mM H_2O_2 for 30 min at room temperature in the dark before a 1.0 hr treatment with 4 mM GSH. For the nitrosative stressor treatments, reduced GSNOR was treated with 2 mM nitrosocysteine (CysNO) for 1.0 hr at room temperature in the dark before a 1.0 hr treatment with 4 mM GSH. Samples were washed twice with 50 mM potassium phosphate pH=7.2 using Amicon-Ultra- 0.5 ml centrifugal filters with a 10 kDa cutoff to remove low molecular weight reagents and salt contaminants.

e. Intact protein mass spectrometry

20 to 40 μM GSNOR was buffer exchanged into 20 mM ammonium acetate and then denatured in 47% methanol + 4% acetic acid (v/v) to a final concentration of 5-12 μM. The solution was infused at a rate of 3μl per min onto an Orbitrap Fusion mass spectrometer (Thermo Fisher) at a spray voltage of 3500 V with a resolution of 60000. Scans were collected in intact protein mode at 1 microscan per sec over the m/z range of 1000-5000. Specifications of the instrument were as follows: RF Lens = 60%, AGC Target = $4.0*10^5$, Sheath gas = 25 abs, Auxiliary gas = 15 abs, Energy = 35 V, Positive ion spray voltage = 3500 V, negative ion spray voltage = 2500 V, Ion transfer tube temperature = 300°C, Vaporizer temperature = 40°C. To determine spectral masses, raw data were processed with Protein Deconvolution 3.0 (Thermo) in isotopically unresolved mode (Manual ReSpect). Ion chromatograms were constructed from the m/z range of 1000-5000, and charge states from +10 to +100 were considered over the mass range of 40,000-45,000 Da. Filters of ±10 ppm, 95% confidence noise reduction, and 1% relative species abundance were applied.

f. Trypsin digestion and LC-MS/MS of peptides

10 μM of GSNOR was dissolved in 50 mM Tris-HCl pH= 8.0. Trypsin was added to a protease: protein ratio of 1:20 (w/w) and incubated at 37°C for 24 hrs. The peptides were dried down in a vacuum-centrifuge and stored at -80°C until further analysis. Data were collected by Dr. Stephen Eyles at the University of Massachusetts, Amherst Mass Spectrometry Facility on an Orbitrap Fusion mass spectrometer. The raw peptide data were processed using Proteomic Discoverer 2.0 (Thermo). The workflow consisted of 1) Spectrum Files 2) Spectrum Selector 3) Scan Event Filter 4) Fixed Value PSM Validator 5) Event Detector 6) Precursor Ions Area Detector. Sequest HT was set to analyze trypsin digestion with a maximum of 4 missed cleavage sites and note

peptide dynamic modifications of S-nitrosation and S-glutathionylation. The precursor mass tolerance was 10 ppm and the fragment mass tolerance was 0.6 Da. The precursor ion area detector was used to quantify the abundance of the specific peptides to determine the relative amount of modified to unmodified peptide.

g. GSNOR activity assay

Enzymatic activity of AtGSNOR was measured in replicates of eight on a 96-well plate using a Biotek Synergy 2 plate reader. Activity was measured by adding 5-10 nM GSNOR in 50 mM potassium phosphate pH 7.2 to 200 μM NADH and 400 μM GSNO as substrate in a final volume of 100 μl and monitoring the decrease in absorbance at 340 nm over 5 min in 5 sec intervals. Relative enzymatic activity was determined by plotting a linear-fit over a 10-15% decrease in absorbance, and comparing it to the linear fit of untreated AtGSNOR as control.

h. Strains of *Saccharomyces cerevisiae*

Wildtype and the SFA1 knockout strain were graciously supplied to us by the John Lopes laboratory at the University of Massachusetts, Amherst. SFA1, AtGSNOR, and the AtGSNOR mutants were introduced into the pRS313 plasmid, a low copy-number plasmid which has a *HIS3* gene to allow for selectivity, and then introduced in the sfa1Δ background by Dr. Damian Guerra.

i. Growth of *S. cerevisiae*

Cells were first grown in synthetic liquid yeast growth medium that had histidine omitted (His^-) to select for the desired plasmid constructs, which all contained a *HIS3* gene (Sup. Fig 1). Cells were grown in standard yeast extract peptone dextrose (YEPD) media (1% yeast extract, 2% peptone, 2% dextrose), except for experiments testing strain phenotype under respiratory

conditions, which used other media, either yeast peptone glycerol (YPG) (1% yeast extract, 2% peptone, 3% (v/v) glycerol) and yeast peptone acetate media (YPAc) (1% potassium acetate, 2% yeast extract, 2% peptone). For all non-temperature stress growth assays, *S. cerevisiae* cells were grown at 30°C in the dark in 10 ml of liquid YEPD media, while being continuously rotated at 100 rpm in a New Brunswick culture wheel. Growth was monitored by checking the optical density of 1 ml of the culture at 600 nm (OD_{600}) at multiple time points. 1 ml of fresh YEPD was added after measurements to maintain a volume of 10 ml. OD_{600} saturation of the yeast cultures in YEPD media occurred at approximately when $OD_{600} \sim 3.0$. For nitrosative stress, either 1 mM GSNO or CysNO was added to the cell culture. For oxidative stress, either 0.5 formaldehyde or hydrogen peroxide was added to the cell culture.

j. *S. cerevisiae* thermotolerance assays

Strains of *S. cerevisiae* were grown overnight to mid-exponential phase ($OD_{600} \sim 1.0$). Samples were then diluted to an OD_{600} of 0.1 and serial diluted 10-fold onto YPD agar plates. For normal growth cells were maintained at 30°C. Continuous heat stress was carried out by growing cells at either 37°C or 42°C. Heat shock was performed by incubating the plates spotted with 10-fold culture dilutions for 1.0 hr at either 42°C or 50°C, while cold shock was performed by incubating the plates for 1.0 hr at 15°C. All plates were imaged after three days of growth.

CHAPTER 3

IN VITRO S-GLUTATHIONYLATION OF GSNOR

a. Introduction

Previous work by Xu et al. (2003) compared the conservation of the amino acid sequences and the high conservation of cysteines in GSNOR. They found that the cysteines not involved in zinc-bonding are 91.0% conserved across plant GSNORs, and that three of these conserved cysteines that were solvent accessible in *A. thaliana* – C10, C271. C370 (Xu et al, 2013). Being highly conserved and solvent accessible suggests that these three cysteines play a role in the regulation and interactions of GSNOR. Solvent-exposed cysteines can be modified by redox PTMs – including oxidation, S-nitrosation, and S-glutathionylation. Previous work has shown than GSNOR can be S-nitrosated by GSNO and other nitric oxide donors (sodium nitroprusside (SNP), S-nitroso-N-acetyl-DL-penicillamine (SNAP), and nitrosocysteine(CysNO)) at those three conserved non-catalytic, non-structural, solvent-accessible cysteine residues (Guerra et al, 2016). The most abundant lower molecular weight nitric oxide donor in cells is S-nitrosoglutathione (GSNO), which is also the substrate of GSNOR (Jensen et al, 1994; Corpas et al, 2013). Interestingly, GSNO can lead to two different PTMs on cysteine residues: S-nitrosation and S-glutathionylation (Giustarini et al, 2005). Experiments described in this chapter were designed to determine if GSNOR can be S-glutathionylated *in vitro*, which conditions lead to S-glutathionylation, the specificity of GSNOR S-glutathionylation, and whether S-glutathionylation has a role in regulating GSNOR enzymatic activity and structural changes.

b. Results

i. *In silico* analysis of GSNOR S-glutathionylation

Preliminary analysis was performed to predict which cysteine residues were most likely to be modified by nitrosation or glutathionylation. S-nitrosation and S-glutathionylation have been hypothesized to occur on cysteine residues with a low pKa (Roos et al, 2013; Broniowska et al, 2014). To estimate the pKa of GSNOR cysteine residues, analysis was first conducted using the PROPKA 2.0 program on two different structures of GSNOR from *A. thaliana*. Analysis was done using the online software PDB2PQR Version 2.0.0 from University of California, San Diego (http://nbcr-222.ucsd.edu/pdb2pqr_2.0.0/). The CHARMM molecular dynamic forcefield and a physiological cytosolic pH of 7.2 were set to analyze the PDB files 3UKO (Crotty et al, 2011) and 4JJI (Crotty et al, 2013). 3UKO has a resolution of 1.4 Å and 4JJI has a resolution of 1.8 Å. The output of predicted pKa values of C10, C271, and C370 are summarized in Table 1. PROPKA analysis of 3UKO gives relatively high pKas for all three of these cysteines. However, the predicted pKa of C370 shifts from 10.26 to 5.76 when analysis is done on 4JJI. The low predicted pKa of C370 suggests that C370 is the most readily glutathionylated cysteine residue. However, that does not mean the other residues cannot be modified by glutathionylation, but rather that the other thiols most likely would need to be modified by either a single oxidation or nitrosation event prior to the glutathionylation.

Table 1. Predicted pKa of conserved, solvent exposed cysteines in GSNOR using PROPKA

This table lists the predicted pKas of C10, C271, and C370 using the PROPKA 2.0 program with a CHARMM molecular dynamic forcefield at a pH of 7.2. Both PBD files of AtGSNOR, 3UKO and 4JJI, were analyzed to determine any pKa differences.

Cysteine	Predicted pKa* of 3UKO	Predicted pKa* of 4JJI
10	10.81	10.66
271	13.59	13.52
370	10.26	5.40

*As determined by PROPKA 2.0 (http://nbcr-222.ucsd.edu/pdb2pqr_2.0.0/)

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ii. GSNOR can be S-glutathionylated *in vitro* by GSNO

As discussed above, GSNO can modify cysteine residues not only by S-nitrosation, but also by S-glutathionylation (Giustarini et al, 2005). S-glutathionylation is the addition of the glutathione (GSH) tripeptide to a reactive thiol of an exposed cysteine residue. To determine if GSNOR could be S-glutathionylated *in vitro*, purified GSNOR was treated with a 100-fold excess of GSNO. One way to observe the appearance of post-translational modifications is by using intact protein mass spectrometry (Hu et al, 2005; Perry et al, 2008). The presence of post-translational modifications on GSNOR was detected by analyzing the shift in the molecular mass and charge states of the different molecular species after 20 μM GSNOR was treated with 2.0 mM GSNO for 1 hr. The purified GSNOR is missing its N-terminal methionine, which shifts the molecular weight to 40565 Da. An example of an ion chromatogram spectrum is shown in Figure 6. The different charge states of GSNOR can be seen from +12 to +21. By expanding a specific charge state, the m/z of the main species can be identified. PTMs can be identified examining spectra for the appearance of new molecular weight species, for example a single modification of S-nitrosation results in mass shift of +29 Da, while a single modification of S-glutathionylation results in a mass shift of +305 Da. As seen in Fig 7A, upon treatment of GSNOR with GSNO, a molecular species appears that corresponds with the predicted shift of S-glutathionylation, with a molecular weight of 40870 Da. Although this modification is in low abundance, it was observed in all spectra of GSNOR treated with GSNO, and in no spectra of untreated GSNOR, which suggests that that the 305 Da shift reflects the addition of GSH. When treated with GSNO, the S-nitrosated species remain, as expected, with shifts of +29 Da (single nitrosation), and +58 Da (double nitrosation). It is worth noting that throughout all experiments there was no consistent

triple nitrosation species observed (shift of +87 Da), as was previously reported by Guerra et al. (2016).

While there are additional peaks in the spectra, these species are observed in all experiments, and are not influenced by treatments to GSNOR. The additional peaks are consistent in biological replicates, with mass shifts of +97, +183, and +374 Da. These species are likely contaminants, as +97 and +183 Da shifts correspond to a N-hydroxysuccinimide (OSu) adduct and a 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) adduct respectively (ABRF, 2017). The +374 Da shift is an unknown contaminant.

To determine the extent of protein modification, the average ion intensities of GSNOR and the modified species were compared from three experiments. As seen in Figure 8, the relative abundance of different GSNOR species is 60.0±3.3% for unmodified, 26.5±2.7% for single nitrosated, 10.7±3.5% for double nitrosated, and 2.8±1.9% for glutathionylated protein. Overall GSNOR S-glutathionylation is in very low abundance in the intact protein mass spectra but is reproducibly detected after treatment of protein with GSNO. These initial findings suggest that GSNOR is not only able to be S-nitrosated by GSNO, but also that it can be S-glutathionylated, albeit to a much lesser extent of overall protein modification.

Figure 6. Mass spectra of intact GSNOR

An example of intact, untreated GSNOR analyzed by protein mass spectrometry. The range of charges states from +12 to +21 can be seen (top). Enlarging a specific charge state, +17 for example (bottom), the m/z of the predominant species can be identified (m/z = 2387 in this case).

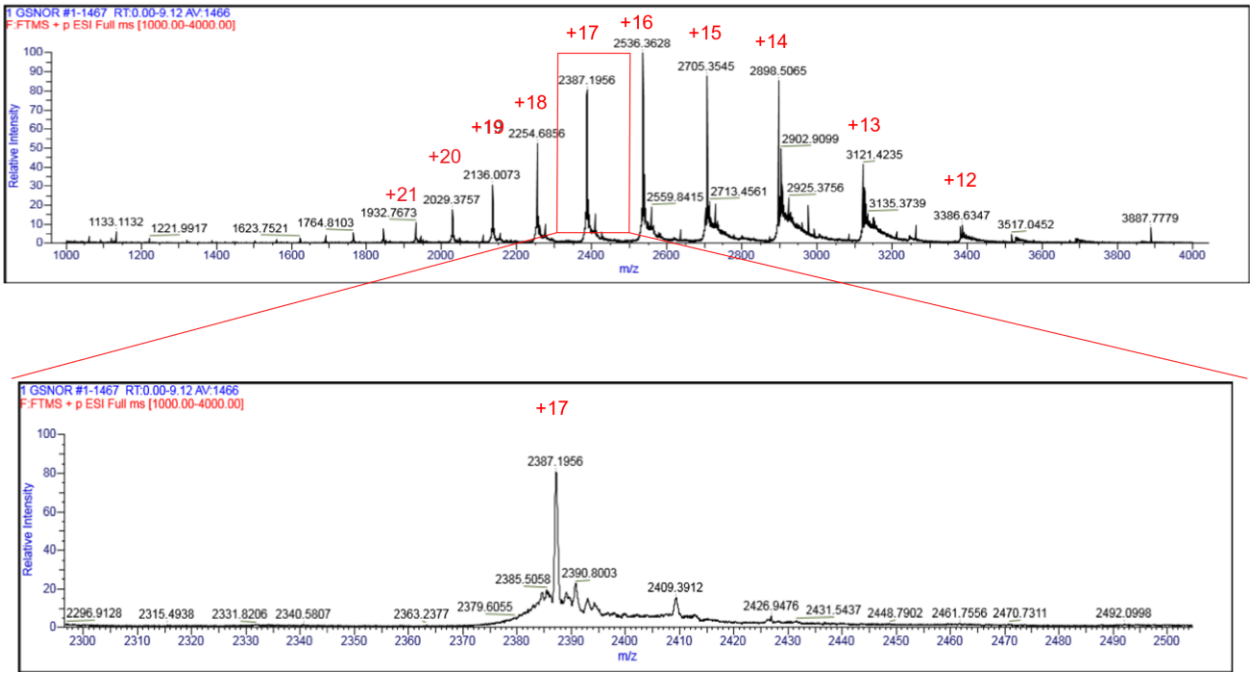
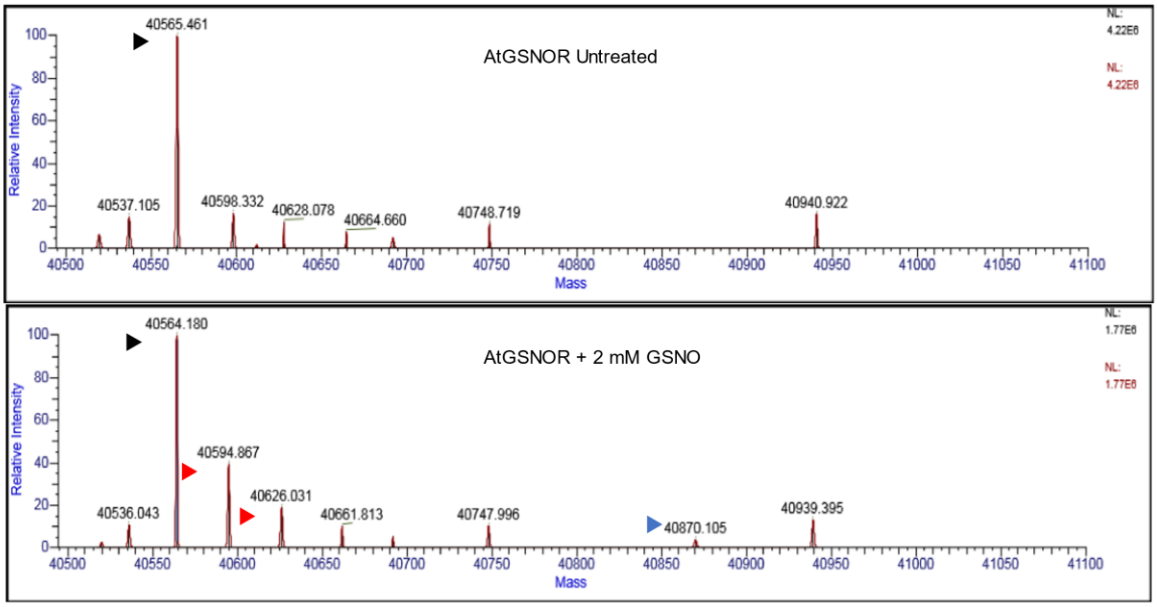


Figure 7. MS spectra of intact GSNOR before and after treatment with GSNO

Deconvoluted masses of AtGSNOR untreated (A, top) and AtGSNOR after treatment with a 100-fold excess of GSNO (A, bottom). Deconvoluted masses of AtGSNOR C271A untreated (B, top) and AtGSNOR C271A treated with a 100-fold excess of GSNO (B, bottom) are shown. The unmodified version of the protein is designated by black arrows, the nitrosated species are indicated with red arrows, and glutathionylated species are indicated with blue arrows. Untreated AtGSNOR and AtGSNOR C271A only have unmodified GSNOR peaks. AtGSNOR treated with GSNO displays peaks of unmodified, single nitrosated, doubled nitrosated, and glutathionylated protein. The AtGSNOR C271A treated with GSNO displays peaks of unmodified, single nitrosated, and double nitrosated protein. This experiment was conducted three separate times using 20 μM GSNOR incubated with 2 mM GSNO for 1 hr. The spectra show the species present within the mass range from 40500 to 41100 Da with a cutoff of 1% relative overall intensity.

A



▶ = Wildtype

▶ = S-nitrosated

▶ = S-glutathionylated

B

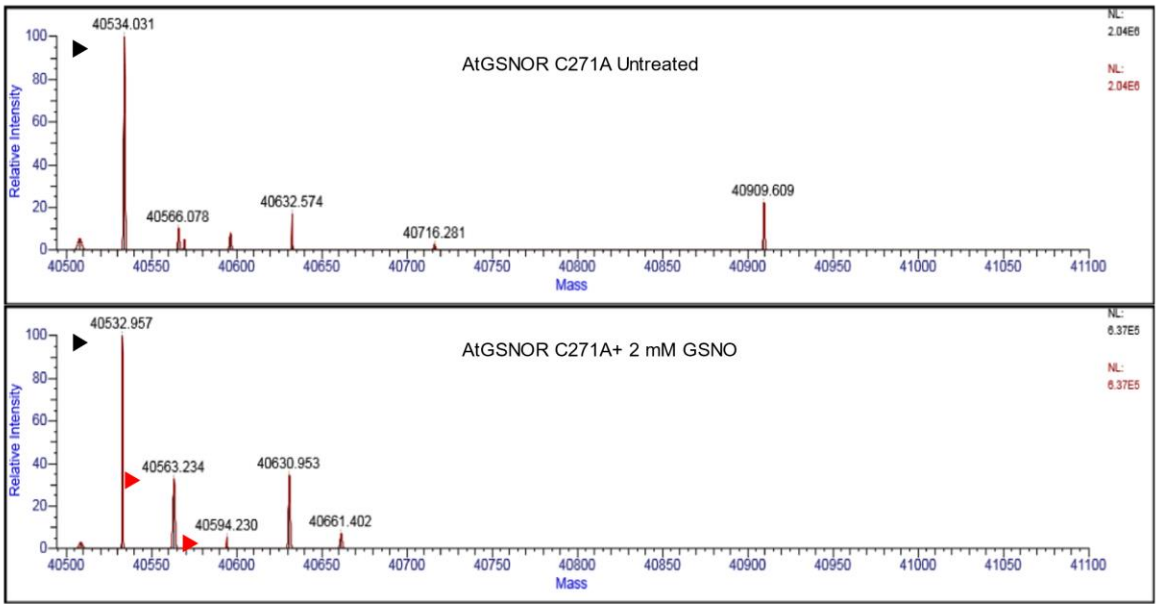
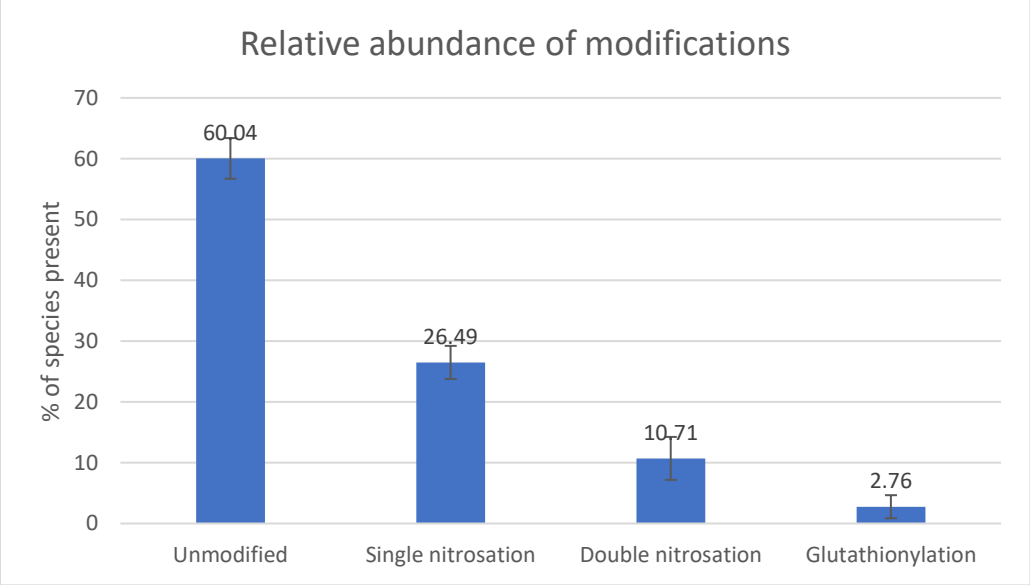


Figure 8. The relative abundance of GSNOR modifications after GSNO treatment

The relative abundance of the different species of GSNOR after treatment with 2 mM GSNO for 1 hr is displayed. After treatment, 60.0±3.3% of GSNOR remained unmodified, while there was 26.5±2.7% and 10.7±3.5 of single and double nitrosation respectively. Triple nitrosation was not detected in any of the experiments where GSNOR was treated with a 100-fold excess of GSNO for 1 hr. Additionally, 2.8±1.9% of GSNOR appears to be glutathionylated. Error bars are two standard deviations from the average ion intensities of the deconvoluted mass spectra from three biological replicates.



iii. Conditions that lead to S-glutathionylation of GSNOR

S-glutathionylation has been shown to be a PTM involved in modulating redox stress (Mieyal and Chock, 2012; Grek, 2013). Specifically, is has been shown to have intermediates that arise from nitrosative and oxidative stress. As seen in Figure 2A, some prospective intermediates contain a nitroso group or a hydroxyl group on the reactive cysteine thiol, which in turn gets replaced by the tri-peptide glutathione. However, not all enzymes have the same cysteine reactivities of the same pathways by which they undergo S-glutathionylation (Grek et al, 2013). Experiments were performed to determine the reaction mechanism(s) by which S-glutathionylation occurs on GSNOR *in vitro*.

In one possible pathway, a reactive thiol interacts with oxidized glutathione (GSSG) which results in S-glutathionylation (Figure 2B). To determine if this is a potential pathway for S-glutathionylation of GSNOR, GSNOR was treated with a 200-fold molar excess of GSSG for different periods of time to see if a S-glutathionylated species formed. The treated GSNOR was then analyzed by intact protein mass spectrometry to check for the predicted +305 Da shift of S-glutathionylation. As seen in Figure 9, no peak that corresponded to S-glutathionylation was observed when GSNOR was treated with GSSG. This experiment was performed in triplicate.

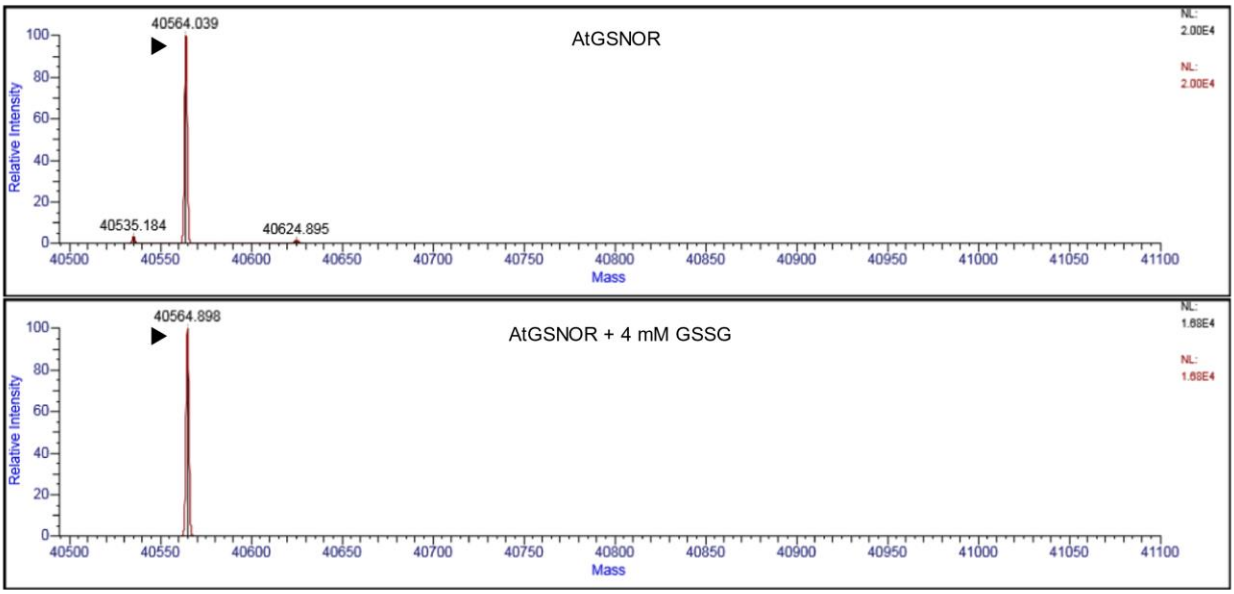
It has been suggested that *in vivo* S-glutathionylation occurs to protect proteins from irreversible oxidation of reactive thiols (Grek et al, 2013). A recent study determined that reactive oxygen species can inhibit GSNOR by binding zinc-coordinating cysteines (Kovacs, 2016). Thus, the next condition that was explored was the oxidative intermediate pathway (Fig. 2B). GSNOR was treated with a 25-fold molar excess of hydrogen peroxide (H_2O_2), had the H_2O_2 removed, and then exposed to a 200-fold molar excess of reduced GSH to determine if S-glutathionylation occurred. After treatment, the GSNOR samples were analyzed by intact protein mass

spectrometry. As seen in Figure 10, there did not appear to be any oxidative modifications corresponding to a mass shift of +16 Da after H_2O_2 treatment. The lack of single oxidation poses a problem if the oxidized cysteine is suspected to be the intermediate species. There was also a significant increase of unknown species, which potentially contaminated the sample and removed oxidative modifications. After treatment with H_2O_2 and GSH, there was no shift of +305 Da, which either suggests that the oxidized hydroxyl-modified intermediates do not lead to S-glutathionylation for GSNOR *in vitro,* or the abundance of unknown species interfered with the oxidative and/or glutathionylation modifications.

Previous work has shown that GSNOR can be S-nitrosated at C10, C271, and C370 (Guerra et al., 2016), suggesting that a pathway of S-glutathionylation might occur through a nitrosative intermediate. To determine if S-nitrosated GSNOR acts as an intermediate for the S-glutathionylation of GSNOR, GSNOR was treated with a 100-fold molar excess of S-nitrosocysteine (CysNO) and then treated with or without GSH. After the treatments, samples were analyzed by intact protein mass spectrometry. As seen in Figure 11, after CysNO treatment single, double, and triple nitrosation were detected. After treatment with CysNO and GSH, there was a mass shift of +305 Da, which suggests that S-glutathionylation occurs through a S-nitrosation intermediate. A summary of the different treatments done to GSNOR and whether they resulted in S-glutathionylation is presented in Table 2.

Figure 9. Intact protein mass spectra of GSNOR before and after treatment with GSSG

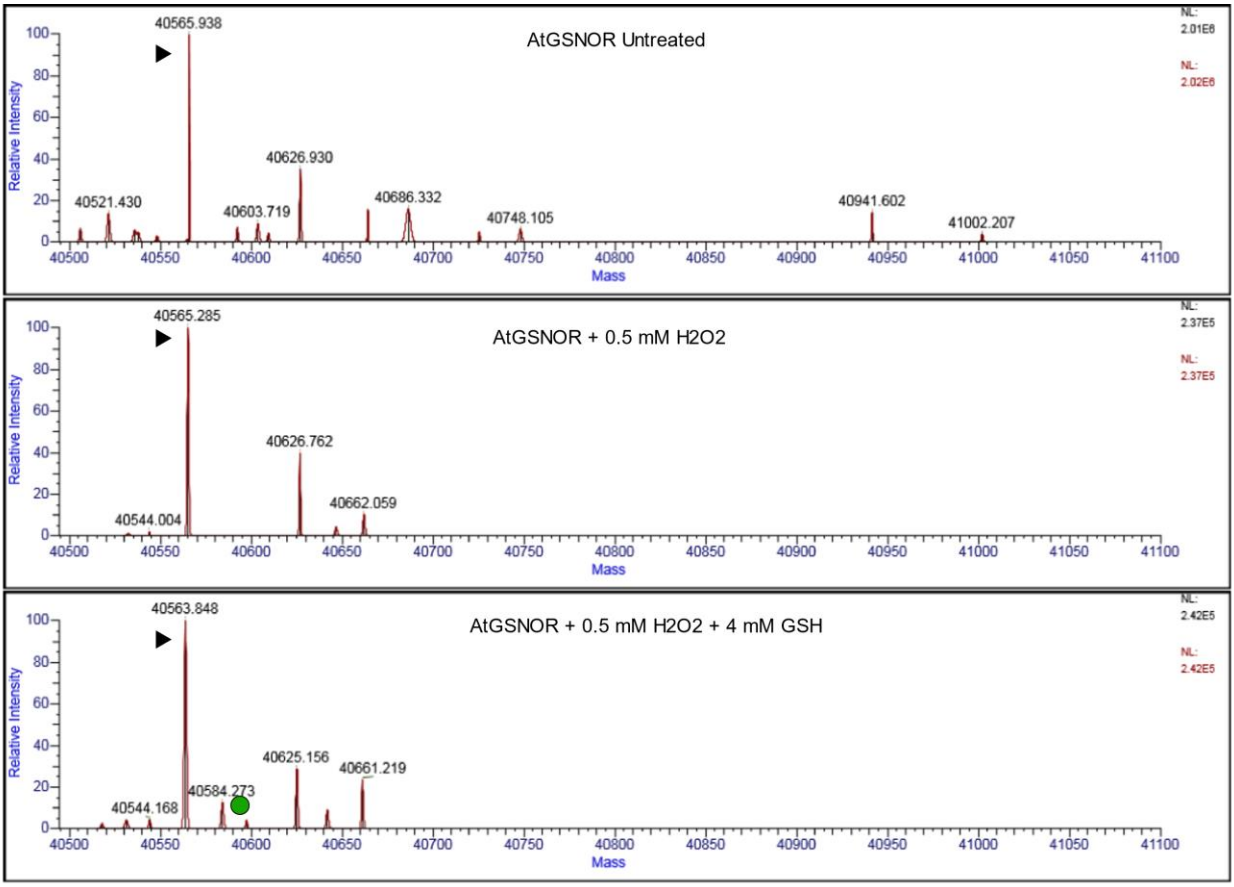
Deconvoluted masses of AtGSNOR untreated (top) and AtGSNOR treated with GSSG (bottom) are shown. The unmodified protein is indicated with black arrows. Only the unmodified protein is present in both the untreated and GSSG treated samples. This experiment was done two separate times using 20 μM GSNOR incubated with 4 mM GSSG for 1 hr. The spectra show the species present within the mass range from 40500 to 41100 Da with a cutoff of 1% relative overall intensity.



▶ = Wildtype

Figure 10. Intact protein mass spectra of GSNOR before and after oxidative treatment

Deconvoluted masses of AtGSNOR untreated (top), AtGSNOR treated with H_2O_2 (middle) and AtGSNOR treated with H_2O_2 and GSH (bottom) are shown. The H_2O_2 was removed by two washes of 50 mM potassium phosphate pH=7.2 prior to GSH treatment. The unmodified protein is indicated with black arrows. Only the unmodified protein is present in both the untreated and treated samples. Possible double oxidation can be seen in the sample treated with H_2O_2 and GSH (bottom, green circle). The lack of oxidation after treatment with H_2O_2 suggests an experimental error occurred. This experiment was performed three separate times using 20 μM GSNOR incubated with 0.5 mM H_2O_2 for 30 min. The spectra show the species present within the mass range from 40500 to 41100 Da with a cutoff of 1% relative overall intensity.



▶ = Wildtype

● = Possible oxidation

Figure 11. Intact protein mass spectra of GNSOR before and after nitrosation treatment

Deconvoluted masses of AtGSNOR untreated (above), AtGSNOR treated with CysNO (middle) and AtGSNOR treated with CysNO and GSH (below) are shown. The CysNO was removed by two washes of 50 mM potassium phosphate pH=7.2 prior to GSH treatment. The unmodified version of the protein is designated by black arrows, the nitrosated species are designated with red arrows, and glutathionylated species are designated with blue arrows. As displayed, S-glutathionylation occurred in the sample that is exposed to both CysNO and GSH. This experiment was performed three separate times using 20 μM GSNOR incubated with 2 mM CysNO for 1 hr. The spectra show the species present within the mass range from 40500 to 41100 Da with a cutoff of 1% relative overall intensity.

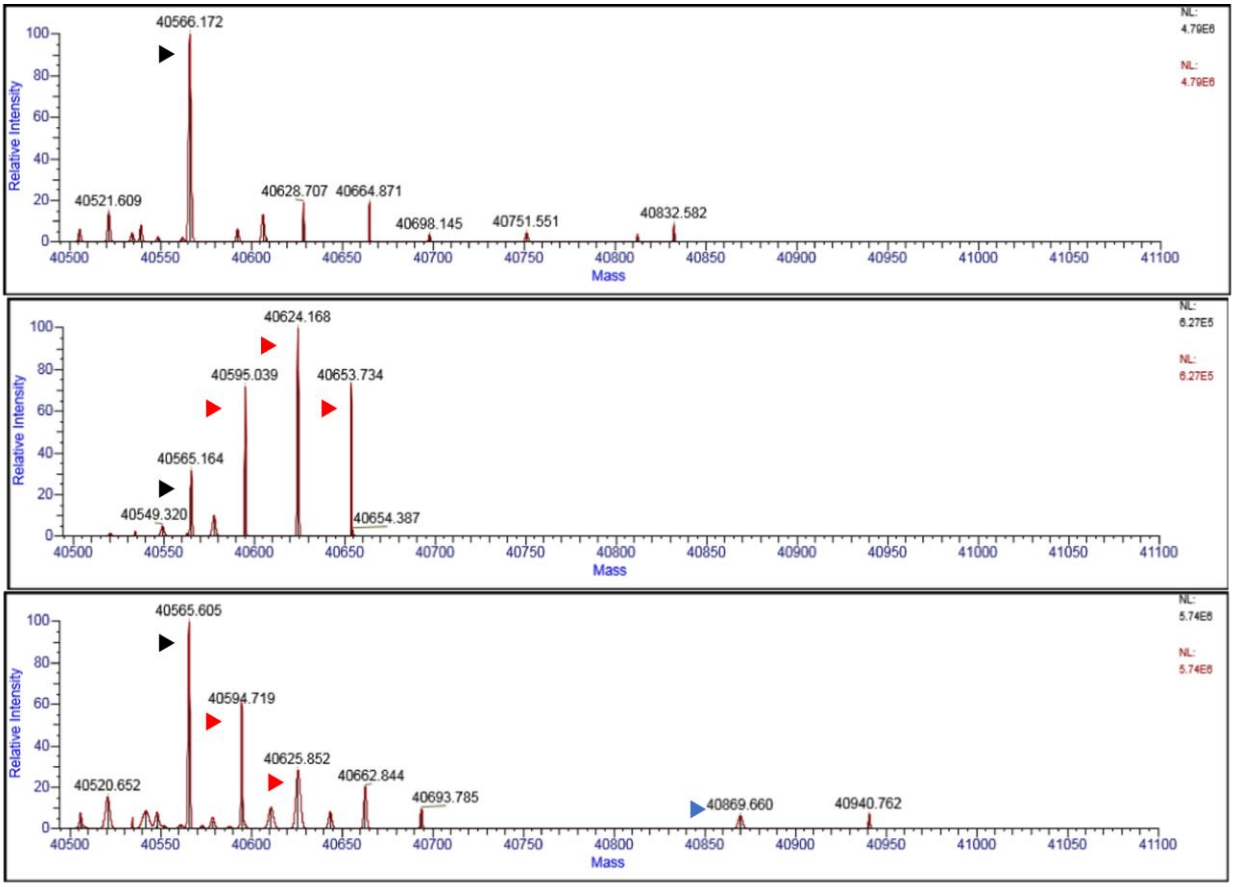


Table 2. Treatments that resulted in S-glutathionylation *in vitro*

This table displays the different treatments done to GSNOR and whether those treatments induced glutathionylation, as determined by intact protein mass spectrometry. As shown, only the GSNO treated and the nitrosative stress plus GSH treatment displayed S-glutathionylated GSNOR as determined from intact protein mass spectrometry.

Treatment	**S-glutathionylation**
GSNO	Yes
GSSG	No
Oxidative Stressor (H_2O_2)	No
Oxidative Stressor and glutathione (H_2O_2 + GSH)	No
Nitrosative Stressor (CysNO)	No
Nitrosative Stressor and glutathione (CysNO + GSH)	Yes

iv. Residue specificity of S-glutathionylation

Previously Guerra et al. (2016) hypothesized that S-nitrosation predominantly occurred on cysteine 370 (C370), so it was of interest to determine on which cysteine S-glutathionylation occurred – whether it was predominantly also C370, or a different residue. To determine which cysteine residue became modified by S-glutathionylation, samples of GSNOR were either untreated, treated with GSNO, treated with solely a nitroso donor or treated with both a nitroso donor and GSH for 3 hr, trypsin digested, and then analyzed by tandem mass-spectrometry to identify which peptide had been modified by S-glutathionylation. The mass spectral data were analyzed using Proteomic Discoverer 2.0. Each of the peptides containing C10, C271, and C370 could be identified and their different length and amino acid sequence allowed for direct comparison between unmodified peptide and the S-glutathionylated peptide.

Four different reactions were performed with GSNOR to elucidate which cysteine residue was susceptible to S-glutathionylation, as listed in Table 3. Reactions 1 (reduced protein) and 3 (treatment with a nitroso donor only) were not expected to induce S-glutathionylation. The other two reactions, 2 (treatment with GSNO) and 4 (treatment with a nitroso donor and GSH), were predicted to induce S-glutathionylation, as previously seen in the intact protein mass spectrometry results. Once the GSNOR was subjected to the different treatments, the protein was trypsin digested, and subjected to tandem mass spectrometry.

As seen in Figure 12, the relative abundance of the different peptides of interest, either unmodified or glutathionylated were compared. Note that S-nitrosation could not be seen on any peptides after collecting MS/MS data. C271 appears to be highly modified by S-glutathionylation in contrast of C10 and C370 after exposure to treatments 2 or 4. After three separate experiments, two of which involved 30 min of their respective treatments and one using

a 3 hr treatment, neither C10 nor C370 were consistent in the level of S-glutathionylation detected, while C271 was consistently highly S-glutathionylated. Both experiments that had a 30 min treatment had a higher level of S-glutathionylation for C10 and C370, but only the experiment that had the 3 hr treatment gave a quantifiable relative ion intensity from the Proteomic Discoverer 2.0 software.

After deconvolving the data from the 3 hr experiment, it appeared that the primary target of S-glutathionylation was the thiol group of C271. There appeared to be minor S-glutathionylation of C10 and C370, but to a much lesser extent. The only peptide that was consistently recovered as S-glutathionylated, and at a high percentage, was the peptide containing C271. The different LC-MS/MS spectra are shown in Figure 13, showing the differences in the b and y ion distribution of the peptide containing C271 after two different glutathionylation inducing treatments. The spectral differences between the unmodified and glutathionylated peptides give confirmation that the peptide containing C271 is indeed S-glutathionylated.

To confirm that C271 is the primary target for S-glutathionylation, GSNOR C271 was mutated to alanine (GSNOR C271A). If C271 was indeed the target of S-glutathionylation, then the GSNOR C271A should not be S-glutathionylated. GSNOR C271A was treated with GSNO, which S-glutathionylated wildtype GSNOR, and then analyzed by intact protein mass spectrometry. GSNOR C271A was treated with GSNO under the same conditions as the wildtype protein and, as seen in Figure 6B, after deconvolving the spectra there is no peak of S-glutathionylation corresponding to a +305 Da shift of GSNOR C271A. These data reinforce the conclusion that S-glutathionylation primarily occurs on C271.

Figure 12. Relative abundance of unmodified versus glutathionylated peptides after MS/MS analysis

The abundance of unmodified to S-glutathionylated peptides is compared after analyzing MS/MS data. Comparisons between abundance of peptides was done using the relative ion intensity of the respective peptide. Both untreated (A) and CysNO (C) treated samples exhibited only unmodified peptides. After treatment with GSNO (B), C271 was highly S-glutathionylated, and C10 and C370 were slightly S-glutathionylated. After treatment with both CysNO and GSH (D), the same trend of C271 being highly S-glutathionylated and low levels of S-glutathionylation on C10 and C370 were detected. This experiment was done once with a 3 hr treatment and twice with a 30 min treatment. The experiments with 30 min treatments did not give confident ion intensity results, so the data have been omitted from this figure.

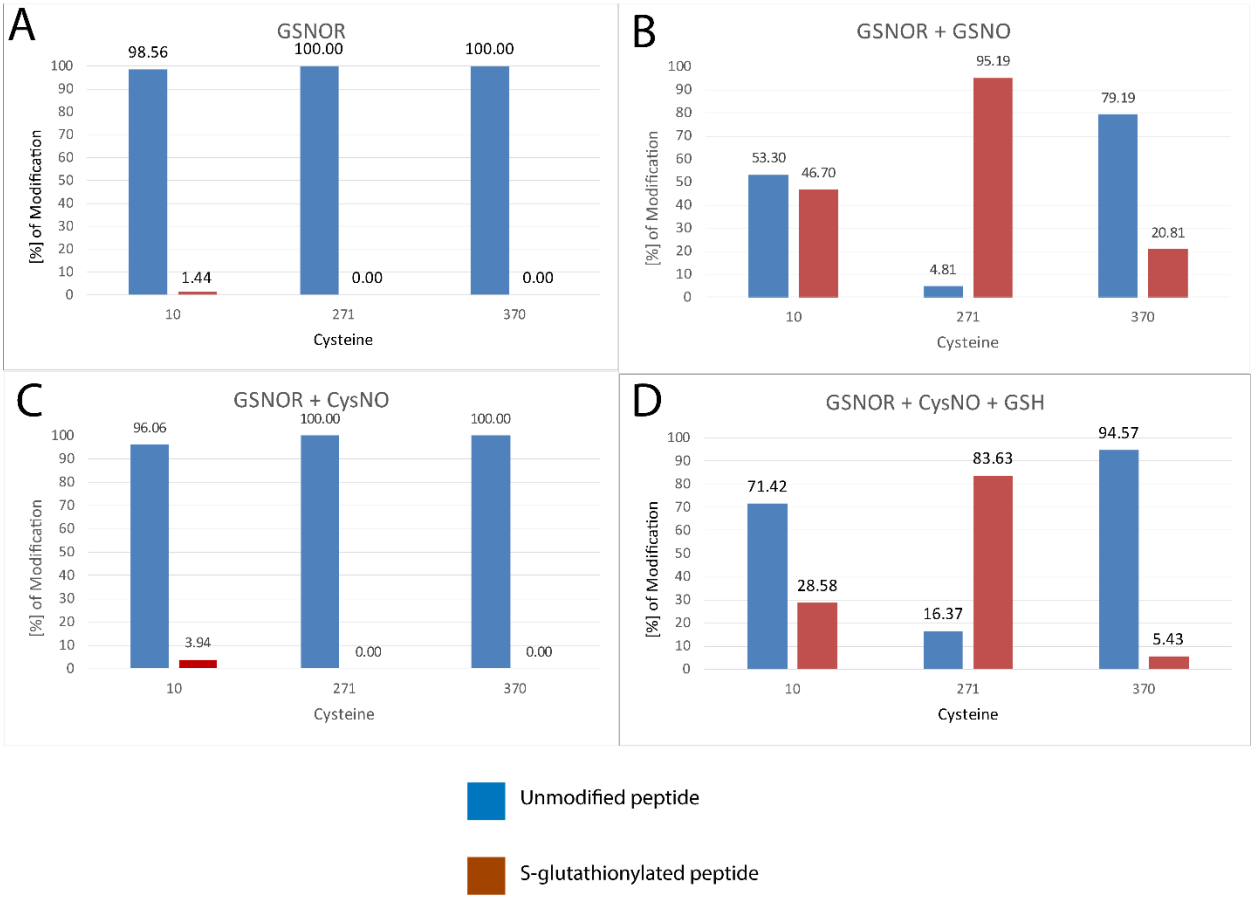
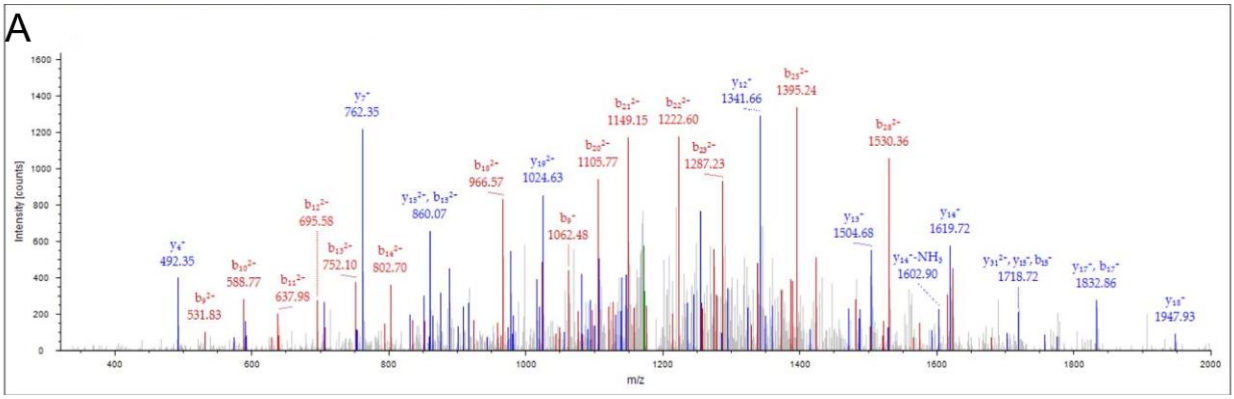
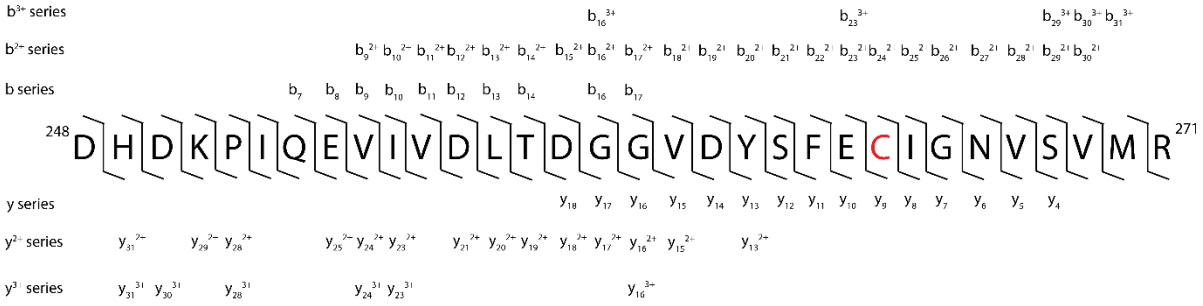
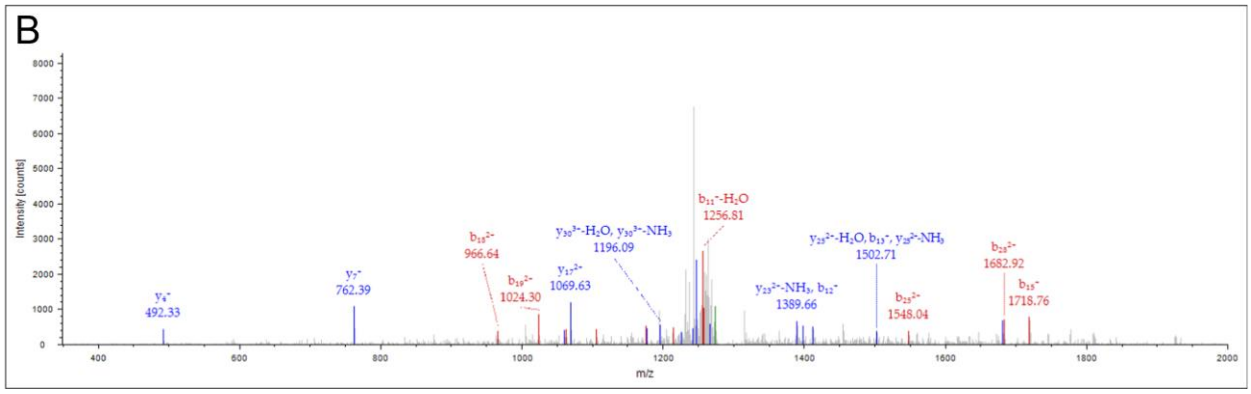


Figure 13. MS^2 Spectra of the unmodified and glutathionylated peptide containing C271

MS/MS spectra of the peptide ^{248}DHDKPIQEVIVDLTDGGVDYSFECIGNVSVMR^{279} which contains C271 (in red). The unmodified peptide spectrum (A) and the S-glutathionylated spectrum (B) were collected after treatment with 2 mM GSNO for 3 hr. The unmodified peptide spectrum (C) and the S-glutathionylated spectrum (D) were collected after treatment with 2 mM CysNO and 4 mM GSH for 3 hr. The respective b and y ion distribution is below every spectra. Analysis of the differences between the b and y ions from the four different spectra support the conclusion that this peptide is S-glutathionylated.







b3+ series

b2+ series b_{18}^{2+} b_{19}^{2+} b_{20}^{2+} b_{25}^{2+} b_{28}^{2+}

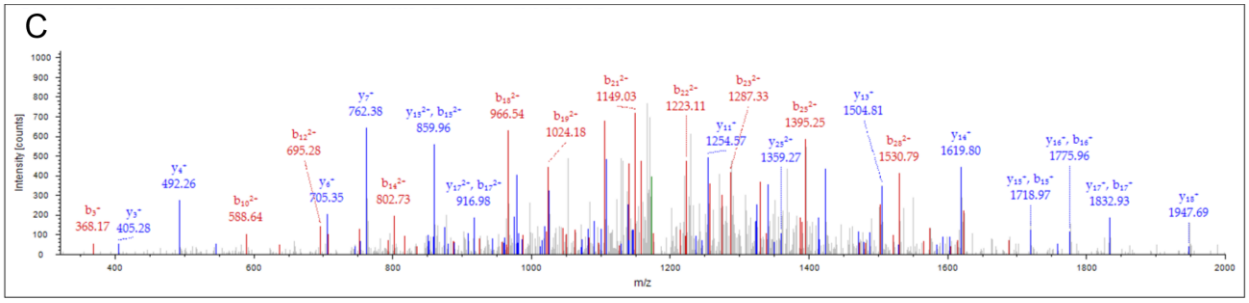
b series b_9 b_{10} b_{11} b_{12} b_{13} b_{15}

^{248}D H D K P I Q E V I V D L T D G G V D Y S F E C I G N V S V M R^{271}

y series y_7 y_4

y2+ series y_{28}^{2+} y_{23}^{2+} y_{19}^{2+} y_{17}^{2+}

y3+ series y_{31}^{3+}



b3+ series b_{23}^{3+} b_{26}^{3-} b_{28}^{3+} b_{29}^{3+} b_{31}^{3-}

b2- series b_9^{2-} b_{10}^{2-} b_{11}^{2+} b_{12}^{2+} b_{13}^{2+} b_{14}^{2-} b_{15}^{2-} b_{16}^{2-} b_{17}^{2-} b_{19}^{2-} b_{20}^{2-} b_{21}^{2-} b_{22}^{2-} b_{23}^{2-}

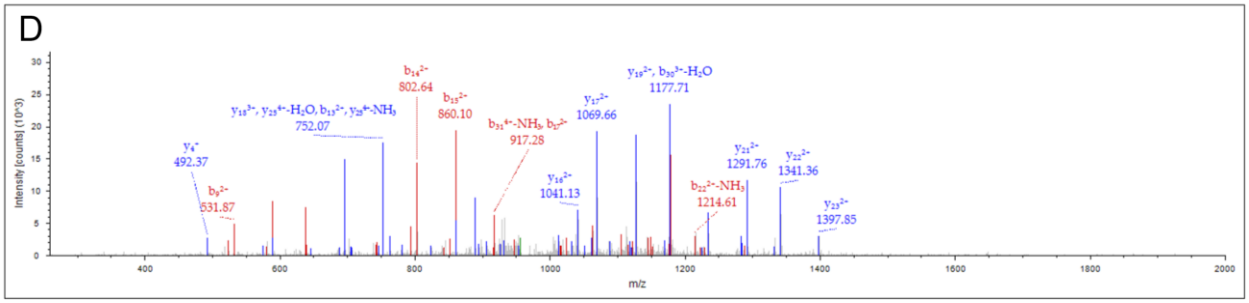
b series b_6 b_9 b_{10}

^{248}D H D K P I Q E V I V D L T D G G V D Y S F E C I G N V S V M R^{271}

y series y_9 y_7 y_6 y_4

y2- series y_{23}^{2+} y_{22}^{2+} y_{21}^{2+} y_{20}^{2-} y_{19}^{2+} y_{18}^{2+} y_{17}^{2+} y_{16}^{2+} y_{15}^{2-} y_{13}^{2+} y_{12}^{2+} y_{11}^{2+} y_{10}^{2+}

y3- series y_{28}^{3-} y_{27}^{3-} y_{26}^{3-} y_{23}^{3-} y_{22}^{3-} y_{21}^{3-} y_{20}^{3-} y_{18}^{3-}



b^{3+} series b_{23}^{3+} b_{27}^{3+} b_{28}^{3+} b_{29}^{3+} b_{30}^{3+} b_{31}^{3+}

b^{2+} series b_{10}^{2+} b_{11}^{2+} b_{12}^{2+} b_{13}^{2+} b_{14}^{2+} b_{15}^{2+} b_{16}^{2+} b_{17}^{2+} b_{18}^{2+} b_{19}^{2+} b_{20}^{2+} b_{21}^{2+} b_{22}^{2+} b_{23}^{2+} b_{24}^{2+} b_{25}^{2+} b_{26}^{2+} b_{27}^{2+} b_{28}^{2+} b_{29}^{2+} b_{30}^{2+} b_{31}^{2+}

b series b_3 b_6 b_7 b_8 b_9 b_{10} b_{11} b_{12} b_{13} b_{14} b_{15} b_{16} b_{17}

248 D H D K P I Q E V I V D L T D G G V D Y S F E C I G N V S V M R 271

y series y_{18} y_{17} y_{16} y_{15} y_{14} y_{13} y_{12} y_{11} y_{10} y_9 y_8 y_7 y_6 y_4 y_3

y^{2+} series y_{29}^{2+} y_{28}^{2+} y_{27}^{2+} y_{26}^{2+} y_{25}^{2+} y_{23}^{2+} y_{21}^{2+} y_{20}^{2+} y_{19}^{2+} y_{18}^{2+} y_{17}^{2+} y_{16}^{2+} y_{15}^{2+}

y^{3+} series y_{31}^{3+} y_{30}^{3+} y_{28}^{3+} y_{27}^{3+}

Table 3. Treatments of GSNOR prior to trypsin digestion and LC-MS/MS

This table lists the different GSNOR treatments done for either 30 min or 3 hr at room temperature prior to trypsin digestion and LC-MS/MS analysis.

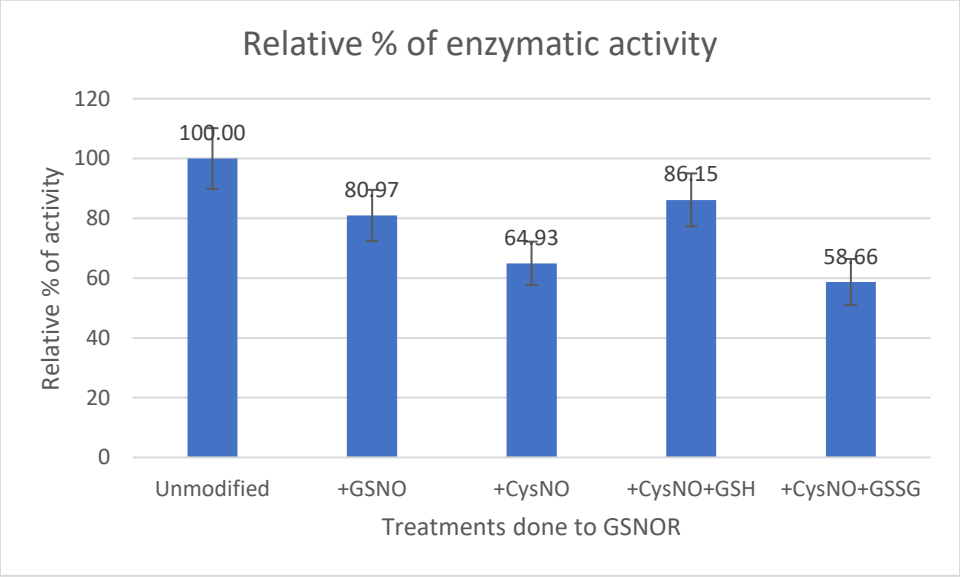
Sample #	**Treatment of 20 µM GSNOR**
1	Untreated
2	2 mM GSNO
3	2 mM CysNO
4	2 mM CysNO + 4 mM GSH

v. S-Glutathionylation impacts GSNOR enzymatic activity

Previously Guerra et al. (2016) showed that S-nitrosation was detrimental to the activity of GSNOR. As shown in Figure 14, GSNOR that is treated to cause glutathionylation (and consequently reduced nitrosation) shows increased enzymatic activity, compared to protein that is only nitrosated. However, it is still unclear if S-glutathionylation impacts GSNOR activity directly. GSH appears to de-nitrosate GSNOR and, according to the intact protein mass spectrometry experiments, the S-glutathionylation only occur in very low abundance relative to the other species present. In the presence of CysNO, a strong nitrosative agent, GSNOR activity decreased to ~65%. However, in the presence of CysNO and GSH, GSNOR activity only decreased to ~85%, similar to the GSNO treatment. This result suggests that GSH acts as a buffer to limit nitrosation of GSNOR, thus limiting the decrease in GSNOR activity in the presence of nitrosative stress. This opens the possibility that GSH and S-glutathionylation do not only act to limit over oxidation *in vivo*, but potentially are able to limit S-nitrosation as well. This pathway needs to be further explored but can potentially be another mechanism able to modulate redox stress due to nitrosative agents.

Figure 14. Relative GSNOR enzymatic activity after different treatments

The relative enzymatic activity of GSNOR in reducing GSNO was assayed after the enzyme was subjected to different treatments. Setting untreated GSNOR activity as 100%, the GSNO treated sample exhibited 80.9±8.5% total activity, while the CysNO treated sample exhibited 64.9±7.2% activity. In the presence of GSH, the CysNO sample regained activity to 86.1±8.8% while the GSSG treated sample showed no recovery of activity and remained at 58.6±7.7%. Error bars are twice the standard deviation from the average of eight replicates. All treatment reagents were removed before assaying activity.



c. Discussion

Based of analysis by intact protein mass spectrometry, a mass shift corresponding to S-glutathionylation (+305 Da) occurred when GSNOR was treated with 2 mM GSNO for 1 hr. However, after the GSNO treatment the overall abundance of the S-glutathionylated species was very low with only 3% of total protein appearing to be glutathionylated. Interestingly, when trypsin digested and analyzed by LC-MS/MS, the peptide containing C271 was highly glutathionylated after both 30 min and 3 hr of treatment with either GSNO or CysNO and GSH. The LC-MS/MS data appear to contradict the intact protein mass spectrometry data. If C271 is determined to be highly S-glutathionylated, the single S-glutathionylated species of GSNOR should be more abundant than 3% of total protein. One possible explanation is that the S-glutathionylation of C271 is disrupting the ionization of intact GSNOR and the S-glutathionylation is not effectively detected by intact protein mass spectrometry using the current settings. By changing the spray voltage and gas settings, it is possible that a higher abundance of S-glutathionylated GSNOR could be detected.

It appears that S-glutathionylation occurs through a nitrosative intermediate. Treatment with GSNO or CysNO and GSH both led to a detectible mass shift of +305 Da using intact protein mass spectrometry. However, the direct interaction between the nitrosated species and glutathione is still unclear. Data shows that the presence of glutathione limits the nitrosation of GSNOR, but it is unclear whether GSH acts by de-nitrosating directly, or if the GSH glutathionylates GSNOR through the nitrosated intermediate to limit nitrosation by blocking thiols or causing a structural change.

Whether GSNOR can be S-glutathionylated through an oxidative intermediate is still unknown. Based on the Kovacs et al. (2016) study, AtGSNOR is prone to oxidative damage and

modifications when exposed to H_2O_2, specifically on zinc-coordinating cysteines C47 and C177. If that was indeed the case, the intact protein mass spectrometry analysis of the H_2O_2 treated samples should have exhibited more oxidative modifications, but in each biological replicate the oxidative modifications were sparse. The lack of oxidative modifications makes it difficult to reach a conclusion on whether GSNOR can or cannot be glutathionylated through the oxidative intermediate pathway. Interestingly, GSNOR from different species behave differently to oxidative stress. AtGSNOR demonstrates a decrease in activity, while the activity of GSNOR from *Chlamydomonas reinhardtii* increases in the presence of H_2O_2. (Zaffagini, personal communication).

CHAPTER 4

PHENOTYPIC ANALYSIS OF *SACCHAROMYCES CEREVISIAE* SENSITIVE TO FORMALDEHYE 1 (*SFA1*) GENE KNOCKOUTS

a. Introduction

Previous work with *A. thaliana* has shown that the enzyme GSNOR is important for maintaining a functional and healthy phenotype when plants are subjected to heat and/or nitrosative stress (Lee et al, 2008; Xu et al, 2015). It has also been shown *in vitro* that the three conserved cysteines, C10, C271, and C370, of GSNOR from *A. thaliana* (AtGSNOR) can be modified by S-nitrosation, which has an impact on enzymatic activity (Guerra et al, 2016). By using *S. cerevisiae*, a simpler eukaryotic organism, which has a single GSNOR gene, Sensitive to Formaldehyde 1 (*SFA1*), it is possible to explore the role that those three evolutionarily conserved cysteines (Figure 3) have *in vivo*. By using different treatments to cell cultures of *S. cerevisiae*, a phenotypic difference was observed between wildtype and a SFA1 knockout (sfa1Δ) strain of *S. cerevisiae*. To determine the importance of the conserved cysteine residues, AtGSNOR and AtGSNOR cysteine to alanine mutants were introduced into the sfa1Δ strains to see if the mutants could complement sfa1Δ and which cysteines may have a role in regulating GSNOR activity *in vivo*. This chapter will first describe experiments to determine if there are phenotypic differences between wildtype and sfa1Δ *S. cerevisiae*, and whether sfa1Δ phenotypes can be complemented by introducing AtGSNOR into the mutant. Further experiments were performed to determine if, and which, of the three cysteine residues have a role *in vivo* using cysteine to alanine mutants of AtGSNOR.

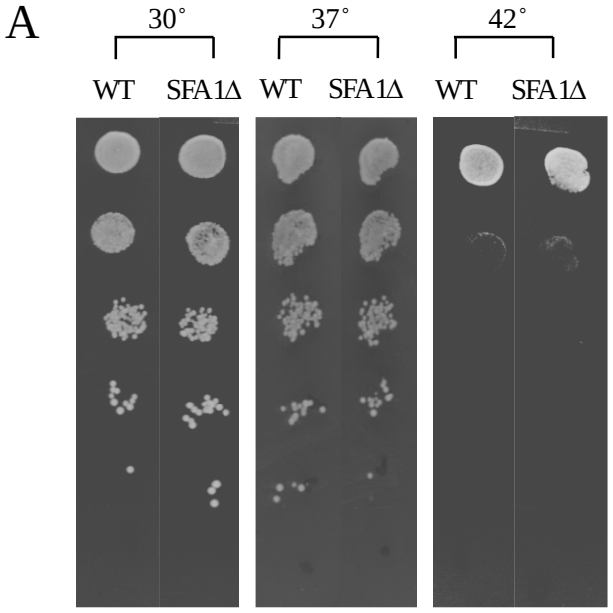
b. Results

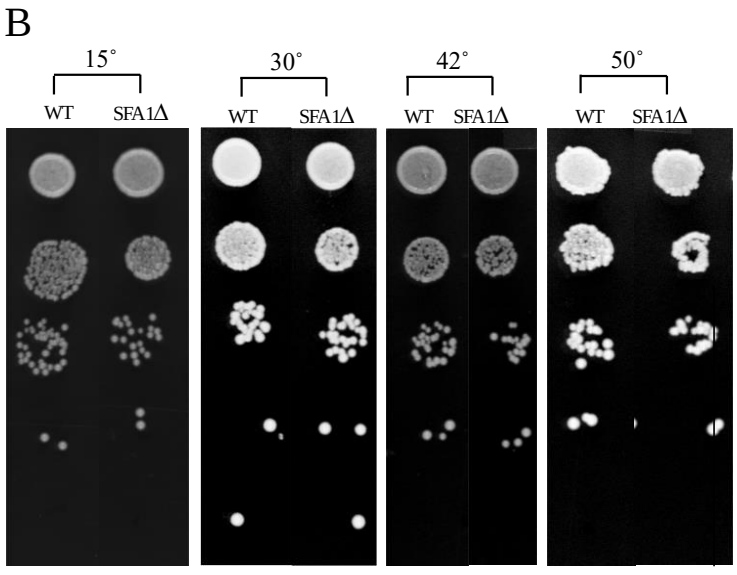
i. Temperature stress of wildtype and sfa1Δ

One of the predominant phenotypes of the *A. thaliana hot5-2* (null for GSNOR) mutant is an increase in heat sensitivity (Lee et al, 2008). To determine if the same phenotype is seen in GSNOR null *S. cerevisiae*, wildtype and sfa1Δ yeast cells were subjected to temperature stress and their growth was monitored. Two different methods of temperature stress were employed to test for a possible temperature sensitive phenotype: continuous temperature stress and brief temperature shock. Both methods were performed by collecting mid-log phase cells, diluting them to an equal optical density (OD_{600}), and then introducing the stress. The first method of subjecting cells to a continuous temperature stress resulted in a decrease in viability as the temperature increased, but there was no difference in phenotype between the wildtype and sfa1Δ cells (Fig 15A). The second method used was to subject the cells to a 1.0 hr temperature shock and then continuing growth at the standard temperature of 30°C. The longer the cells were subjected to the stress, the greater the decrease in viability, but consistent with the first test there was no differences between the wildtype and sfa1Δ cells (Fig. 15B). Using both methods, it appears that the lack of SFA1 does not affect sensitivity to different types of temperature stress.

Figure 15. Temperature tolerance assays of wildtype and sfa1Δ

Both wildtype and sfa1Δ cells were diluted to an OD_{600} of 0.1 and serially diluted 10-fold and spotted on YEPD media plates. A) Plates were incubated continuously at either 30°C, 37°C, or 42°C and imaged after three days. B) Plates were incubated for 1.0 hr at the indicated temperatures, and then incubated at 30°C and imaged after three days.



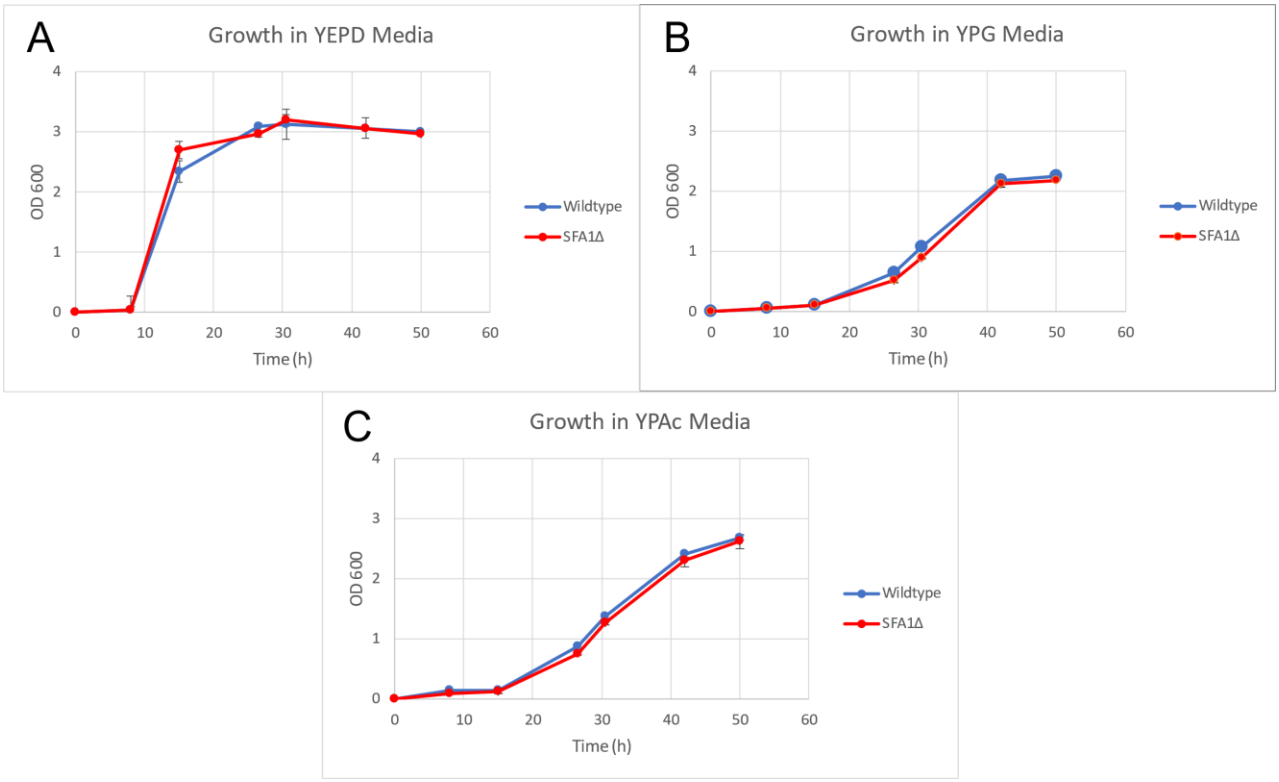


ii. Tests of wildtype and sfa1Δ growth on different respiratory media

S. cerevisiae is normally grown in media that contains glucose to allow glycolysis and the normal respiratory cycle. The standard media, yeast extract peptone dextrose (YEPD), contains glucose (dextrose) as the main carbon source to allow growth with glycolysis under aerobic conditions. However, to eliminate glycolytic formation of ATP and force cells to depend solely on respiration for energy production the main carbon source of the media can be altered. To determine if the enzyme SFA1 has a significant role when *S. cerevisiae* is growing under respiratory conditions, wildtype and sfa1Δ cells were grown in media with different carbon sources. Yeast peptone glycerol (YPG) and yeast peptone acetate (YPAc). Yeast can use three-carbon glycerol or two-carbon acetate as a carbon source, instead of six-carbon dextrose (Gancedo et al, 1968; Lages et al, 1997; Minard and McAlister-Henn, 2009; Orlandi et al, 2013). Cells were cultured in the respective media at the normal growth temperature of 30°C, and OD_{600} was measured over time. As seen in Figure 16A, both wildtype and sfa1Δ have the same growth curve in glucose-based YEPD media. Both YPG (Fig 16B) and YPAc (Fig 16C) media led to a slower growth rate for both wildtype and sfa1Δ compared to YEPD media (Fig 16A). Cells in the YPG and YPAc media also reached a lower saturated OD of ~2.2 and ~2.8, respectively, at stationary phase (differing from the saturated OD of ~ 3.0 in YEPD media) but there was no significant phenotypic difference between the growth of wildtype and sfa1Δ.

Figure 16. Growth of wildtype and sfa1Δ in different types of media

Wildtype and sfa1Δ *S. cerevisiae* were grown in three different types of media with different carbon sources to promote glycolysis of respiration. Growth was measured by OD_{600} at different time points until saturation around OD ~ 3.0. Wildtype is indicated in blue and sfa1Δ is indicated in red. A) Growth in YEPD media B) Growth in YPG media C) Growth in YPAc media. The error bars represent two standard errors away from the mean after the experiment was done in triplicate.



iii. Nitrosative stress of wildtype and sfa1Δ

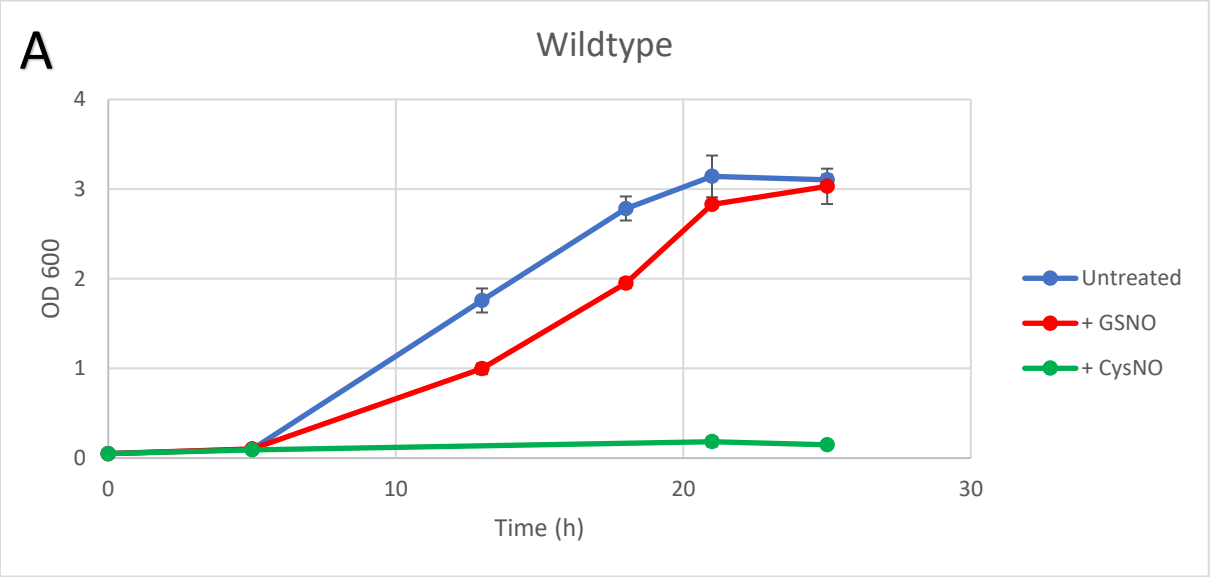
The main substrate of GSNOR is GSNO, the most abundant low molecular weight cellular nitrosative agent, and disruption of GSNOR increases the overall concentration of reactive nitrogen species and abundance of protein S-nitrosothiols in *A. thaliana* and *H. sapiens* (Brzezek, 2014; Barnett and Buxon, 2017). This accumulation of nitrogen species and S-nitrosothiols would have consequences in standard cellular function and growth. Since SFA1 is a homolog of GSNOR, it is suspected that wildtype and sfa1Δ would exhibit a difference in growth phenotype when subjected to nitrosative stress. Both wildtype and sfa1Δ were subjected to either 1 mM GSNO or CysNO, both NO donors, and cellular growth was monitored by OD_{600} over time. Once the nitrosative stress agents were added, the growth patterns of wildtype and sfa1Δ were different than originally expected. As seen in Figure 17, GSNO exposure caused a slower growth phenotype for both wildtype and sfa1Δ, but cells still managed to recover full growth to stationary phase over the course of 25 hrs. However, by the time the GSNO treated samples reached full growth, the CysNO treated samples were still in lag phase growth. The CysNO treated samples had a significantly slower growth than the untreated or GSNO treated samples and took approximately 75 hrs to reach stationary phase (data not shown), but wildtype and sfa1Δ still did not exhibit a significant phenotypic difference in growth patterns.

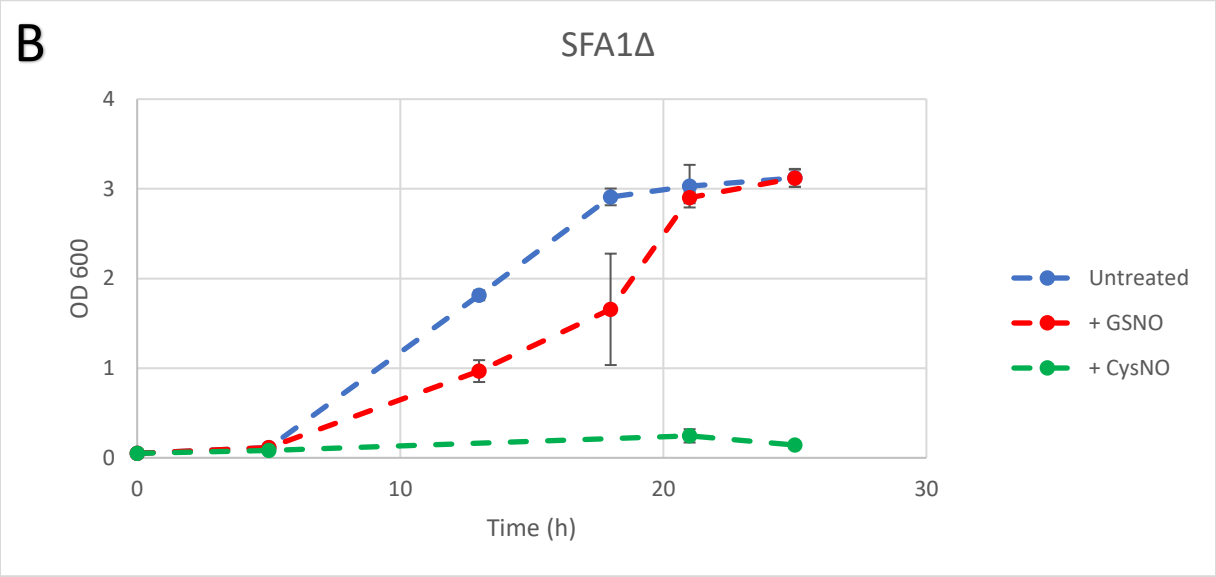
Figure 17. Growth of wildtype and sfa1Δ after nitrosative stress

Wildtype and sfa1Δ cells were either untreated or subjected to nitrosative stress, either 1 mM GSNO or 1 mM CysNO, and growth was monitored by measuring OD_{600} over time. The untreated samples are indicated in blue, the GSNO treated samples are in red, and the CySNO treated samples are in green. The error bars represent two standard errors away from the mean after the experiment was done in triplicate.

A) The growth of wildtype yeast cells (solid lines)

B) The growth of sfa1Δ yeast cells (dashed lines)





iv. Oxidative stress of wildtype and sfa1Δ

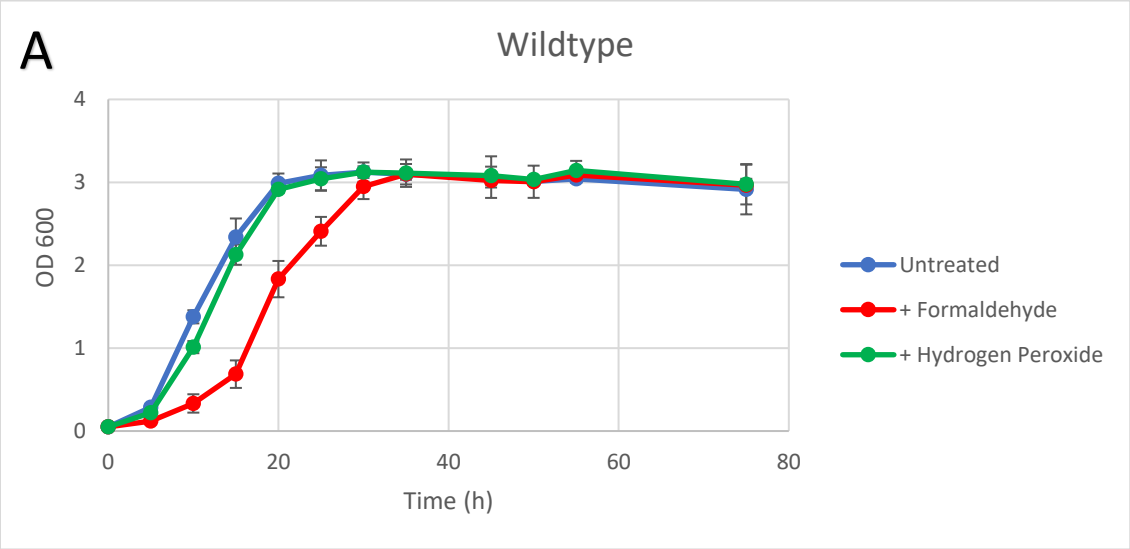
Since SFA1 has been characterized as an enzyme that can oxidize formaldehyde in the presence of glutathione, it is expected that wildtype and sfa1Δ would exhibit a different phenotype when subjected to formaldehyde as shown by Wehner et al (1993). Formaldehyde is a highly reactive aldehyde that can lead to protein oxidation or DNA crosslinking (Whipperman et al., 1999). Formaldehyde can react with glutathione to form hydroxymethylglutathione (HMGSH), which is a substrate for SFA1 in the oxidative direction, converting HMGSH to GSSG in a NAD+-dependent manner (Wehner et al, 1993; Fernandez et al, 2003) The phenotypic differences between wildtype and sfa1Δ in the presence of formaldehyde and another commonly used oxidative agent, hydrogen peroxide (H_2O_2), were explored. Wildtype and sfa1Δ yeast were either treated with 0.5 mM formaldehyde or 0.5 mM $H_2O_2$and growth was monitored by OD_{600} over time (Figure 18). Interestingly for both wildtype (Figure 18A) and sfa1Δ (Figure 18B), there were no growth differences between the untreated and H_2O_2 treated samples. However, there was a difference between wildtype and sfa1Δ growth upon exposure to formaldehyde. Both samples recovered and grew the full stationary phase OD_{600} ~ 3.0 after treatment with 0.5 mM formaldehyde, but wildtype reached that point around the 30-35 hrs, while sfa1Δ took 75-80 hrs to reach stationary phase after formaldehyde exposure.

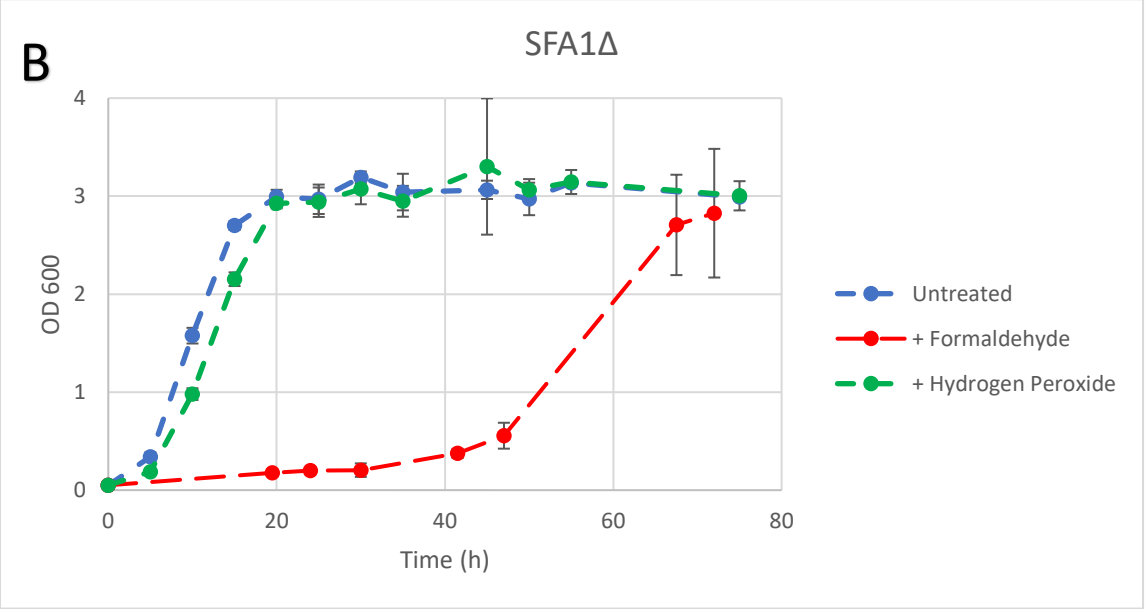
Figure 18. Growth of wildtype and sfa1Δ after oxidative stress

Wildtype and sfa1Δ cells were either untreated or subjected to oxidative stress, either 0.5 mM formaldehyde (FA) or 0.5 mM H_2O_2, and growth was monitored by measuring OD_{600} over time. The untreated samples are indicated in blue, the FA treated samples are in red, and the H_2O_2 treated samples are in green. The error bars represent two standard errors away from the mean from three replicates.

A) The growth of wildtype yeast cells (solid line)

B) The growth of sfa1Δ yeast cells (dashed line)



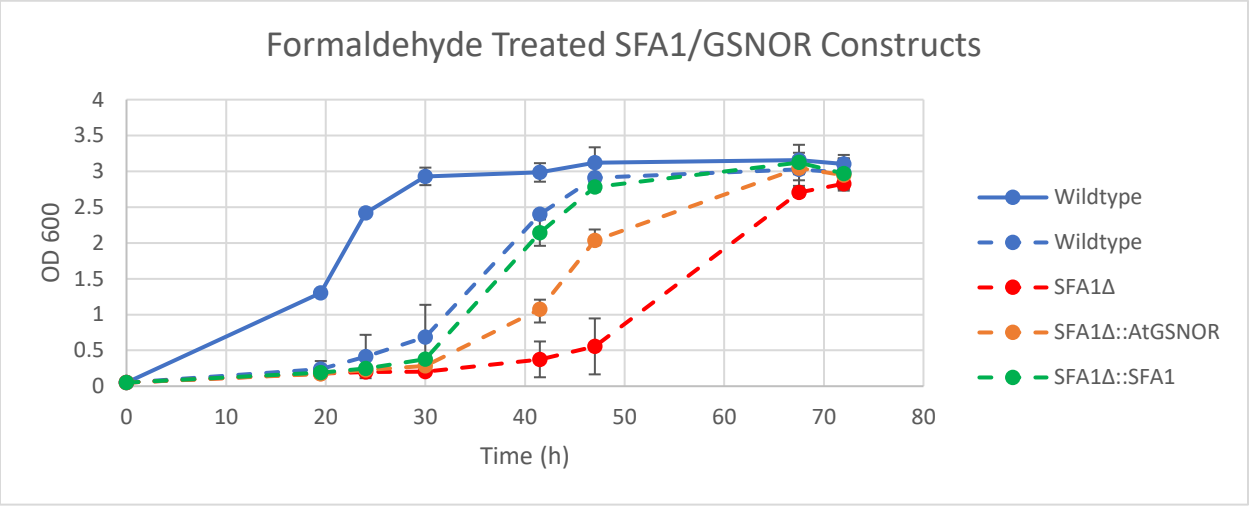


v. The sfa1Δ mutant can be complemented by AtGSNOR

To confirm that the sensitivity to formaldehyde was indeed due to the knockout of SFA1, a pRS313 low-copy plasmid containing SFA1 was introduced into the sfa1Δ (sfa1Δ::*SFA1*) background and cells were subsequently treated with formaldehyde. As seen in Figure 19, the reintroduction of the SFA1 gene restored the ability of cells to recover after exposure to formaldehyde, as seen for wildtype cells. Next, since SFA1 is a homolog of AtGSNOR, it was suspected that AtGSNOR would complement SFA1 activity when introduced into the sfa1Δ background (sfa1Δ::*AtGSNOR*). To ensure that the construct did not lead to any growth defects, growth of the sfa1Δ, sfa1Δ::*SFA1,* and sfa1Δ::*AtGSNOR* cells was monitored under non-stress conditions, and they all demonstrated the same growth as wildtype (data not shown). Once that was established, the sfa1Δ::*AtGSNOR* cells were treated with 0.5 mM formaldehyde, and found to regain partial wild type recovery and growth, albeit not to the extent as wildtype.

Figure 19. Introducing AtGSNOR into sfa1Δ can partially complement the deletion phenotype

Both SFA1 and the AtGSNOR gene were introduced back into the sfa1Δ background in pRS313 (a low-copy CEN plasmid) and treated with 0.5 mM formaldehyde. The controls of untreated wildtype (solid blue line), formaldehyde treated wildtype (dashed blue line), and formaldehyde treated sfa1Δ (dashed red line) were used to check relative growth curves of the different strains. All untreated samples behaved the same as wildtype (data not shown). The sfa1Δ::SFA1 construct (green dashed line) was able to complement the mutant and the cells exhibited the same growth curve as wildtype. The sfa1Δ::AtGSNOR construct partially complemented the mutant and was able to recover from the formaldehyde treatment faster than the sfa1Δ cells, but not as rapidly as wildtype or sfa1Δ::SFA1. Error bars are two standard deviations of three replicates.



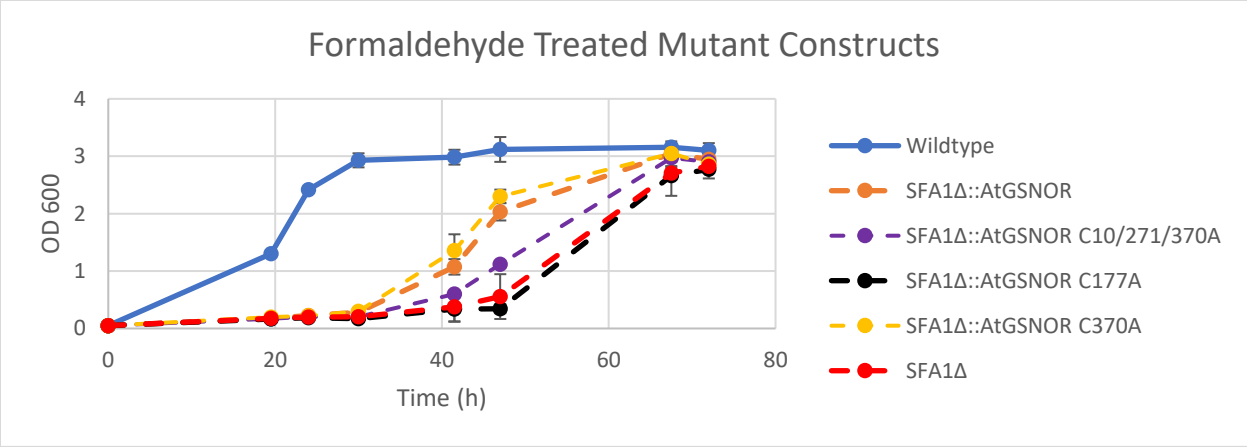
vi. Specific cysteine residues are necessary for full complementation of sfa1Δ

Previous work by Guerra et al. (2016) hypothesized that C370 of AtGSNOR was the most important non-catalytic, non-structural conserved cysteine residue involved in post translational regulation of GSNOR activity. However, recent *in vivo* work suggests that C271 appears to be the most readily nitrosated residue and might have a role in *in vivo* regulation (Ticha et al, 2017). To test this hypothesis, AtGSNOR cysteine to alanine mutants were cloned into a pRS313 plasmid containing a *HIS3* gene, and were introduced into the sfa1Δ cells and selected for in His^- media. Initially, three different cysteine to alanine mutants of AtGSNOR were introduced into the sfa1Δ background: AtGSNOR C370A, AtGSNOR C10/271/370 (triple mutant), and AtGSNOR C177A. The AtGSNOR C177A mutant is catalytically dead, as C177 is a catalytic zinc-binding cysteine required for enzyme activity; AtGSNOR C177A should exhibit the same phenotype as the sfa1Δ cells once exposed to formaldehyde. To ensure that cells carrying the plasmids did not have growth defects under normal conditions, all the strains were originally cultured at 30°C in non-stress conditions and found to all grow the same as wildtype (data not shown). However, phenotypes emerged once the cells carrying the different constructs were treated with formaldehyde. The AtGSNOR construct was regarded as the baseline of full activity and normal growth in the presence of formaldehyde since all the mutants were constructed in AtGSNOR. As seen in Figure 20, the AtGSNOR C177A catalytically dead mutant behaved as expected and demonstrated the same growth pattern as sfa1Δ in the presence of formaldehyde. Interestingly, the strain carrying AtGSNOR C370A also exhibited the same growth pattern as the strain with wildtype AtGSNOR, which was not expected based on the initial hypothesis that C370 plays the most important role for regulation *in vivo*.

To expand on the analysis of the conserved cysteine residues, the triple mutant AtGSNOR C10/271/370A was expressed in sfa1Δ yeast cells and found to support a slower growth rate than wildtype AtGSNOR or the AtGSNOR C370A mutant, but the triple mutant still recovered more rapidly after formaldehyde exposure than sfa1Δ of the AtGSNOR C177A catalytically dead mutant. These data suggest that either C10 or C271, or some combination of conserved cysteine residues, are necessary for full enzymatic activity and formaldehyde detoxification. Alternatively, it is possible that the triple mutant AtGSNOR is less stable in yeast, and therefore less effective at complementation.

Figure 20. Expression of specific AtGSNOR cysteine mutants in yeast fail to complement a sfa1Δ mutant

The wildtype AtGSNOR and cysteine to alanine mutant forms of AtGSNOR were introduced back into the sfa1Δ background. All untreated samples behaved the same as wildtype (blue solid line and data not shown). The sfa1Δ (red dashed line) and the AtGSNOR C177A (black dashed line) catalytically dead mutant behaved in the same manner after exposure to 0.5 mM formaldehyde, failing to recover until much later than wildtype. The wildtype AtGSNOR (orange dashed line) and the AtGSNOR C370A (yellow dashed line) behaved the same after exposure to 0.5 mM formaldehyde. The AtGSNOR C10/271/370A (purple dashed line) triple mutant behaved differently than wildtype AtGSNOR and catalytically dead AtGSNOR, which suggests that C10 and/or C271 are needed to retain the full formaldehyde detoxification activity of AtGSNOR. Error bars are two standard deviations of three replicates.



c. Discussion

There appears to be a very distinct function for the homolog of GSNOR, SFA1, in *S. cerevisiae*. While in other eukaryotes GSNOR has a crucial role in regulation of reactive nitroso species and protein S-nitrosation, which can lead to many downstream effects, SFA1 appears to be essential only for detoxification of formaldehyde through the HMGSH pathway. After subjecting *S. cerevisiae* cells to different types of temperature stress, different nitroso donors, and different oxidative stressors, there appeared to be no difference in a growth phenotype between wildtype and sfa1Δ cells. The only treatment that revealed a significant phenotypic change between wildtype and sfa1Δ cells was treatment with formaldehyde. As SFA1 means "sensitive to formaldehyde" it was expected that the mutant cells would be sensitive to formaldehyde. However, due to the high conservation of SFA1 with GSNOR proteins, the sfa1Δ mutant was expected to exhibit an additional phenotype when subjected to the other stressors, based on results from GSNOR mutants in organisms. Clearly, yeast has additional mechanisms beside GSNOR activity to handle these other stress conditions.

Interestingly, it has been reported that *S. cerevisiae* has another enzyme that primarily deals with nitrosative stress in lag and exponential phase growth, yeast flavohemoglobin1 (YHB1). It was originally thought that YHB1 was mainly involved in pathways that dealt with oxidative stress, but further studies confirmed that YHB1 is involved with managing nitrosative stress (Zhao et al, 1996; Liu et al, 2000). Both SFA1 and YHB1 are localized in the cytosol and it has been reported in YHB1 knockout strains that SFA1 can recover some activity, but it is still unclear how YHB1 and SFA1 complement and work with each other (Cassanova et al, 2004; Li et al, 2011).

However, the difference in phenotype between wildtype and sfa1Δ after formaldehyde exposure proved to be useful to explore the importance of non-catalytic, non-structural, conserved cysteine residues found in AtGSNOR. SFA1 introduced intro the sfa1Δ background restored the ability of cells to recover from the formaldehyde. Also, AtGSNOR introduced to the sfa1Δ background allowed cells to partially recover from formaldehyde treatment. Recovery of formaldehyde treatment from introducing wildtype AtGSNOR allowed testing the importance of conserved cysteine residues *in vivo*. In a non-stress environment, cells carrying both the wildtype and cysteine mutant AtGSNOR proteins displayed no detrimental growth phenotypes. However, some differences emerged when the cells with the mutant constructs were subjected to formaldehyde treatment. Interestingly, and against the initial hypothesis, the AtGSNOR C370A mutant displayed the same phenotype as wildtype AtGSNOR. However, note the previous work by Guerra et al. (2016) examined the GSNO reduction activity, and not the oxidation of hydroxymethylglutathione, which is the mechanism of formaldehyde detoxification. It is still possible that C370 is crucial for GSNO reduction activity and maintaining NO homoeostasis system in *A. thaliana*, although is it not critical for formaldehyde detoxification activity in *S. cerevisiae.*

Although C370 did not seem to have an impact on the formaldehyde detoxification, mutants of the other two conserved cysteines, C10 and C271, exhibited slower growth recovery when subjected to formaldehyde. The AtGSNOR C10/271/370A triple mutant displayed a growth phenotype that was in between that of wildtype AtGSNOR and the catalytically dead AtGSNOR C177A. There are at least three possible explanations for this triple mutant phenotype: 1) C10 or C271 are individually required for the full activity of AtGSNOR in formaldehyde detoxification. 2) Some combination of the conserved cysteines (e.g. both 10 and 271) is required for the full

activity of AtGSNOR for formaldehyde detoxification. 3) At least one of those three conserved cysteine residues (e.g. 370) is required for the full activity of AtGSNOR for formaldehyde detoxification.

By exploring those three different possibilities and the necessary cysteines for full AtGSNOR formaldehyde detoxification, the importance of these three conserved cysteines could be elucidated. It is still unclear if those specific cysteines undergo post-translational modifications, such as S-nitrosation and S-glutathionylation, *in vivo* when subjected to formaldehyde, nitrosative and/or oxidative stress, or if those cysteines are used in some other manner to maintain NO homeostasis during stress.

CHAPTER 5

FUTURE DIRECTIONS

a. Enzymatic S-glutathionylation of GSNOR

The immediate future of this work is the continuation of elucidating how S-glutathionylation can affect the structure and activity of GSNOR. According the intact protein mass spectrometry data, non-enzymatic glutathionylation occurs at low levels *in vitro*. It still needs to be determined if there is an oxidative pathway for the S-glutathionylation of GSNOR. However, potential routes of using enzymes, such as GSTπ, or a higher concentration of GSNO to further glutathionylate GSNOR can be explored to get a larger amount of the S-glutathionylated protein. Once a higher percentage of S-glutathionylated protein can be obtained, analysis could be conducted using circular dichroism to determine if any secondary structure changes arise from S-nitrosation and S-glutathionylation. Further confirmation studies could also be performed to determine if other homologs of GSNOR are S-glutathionylated as well. Further work can be done using anti-GSH antibodies after *in vitro* treatments to analyze potential modifications alongside *in vivo* analysis after plants were subjected to stressors.

b. Elucidating the role of the conserved cysteines in formaldehyde detoxification

The role of conserved cysteines of SFA1 in formaldehyde detoxification also needs to be tested. The current experiments only tested AtGSNOR cysteine to alanine mutants. SFA1 cysteine to alanine mutants have been made to determine the importance of these conserved cysteines in *S. cerevisiae*.

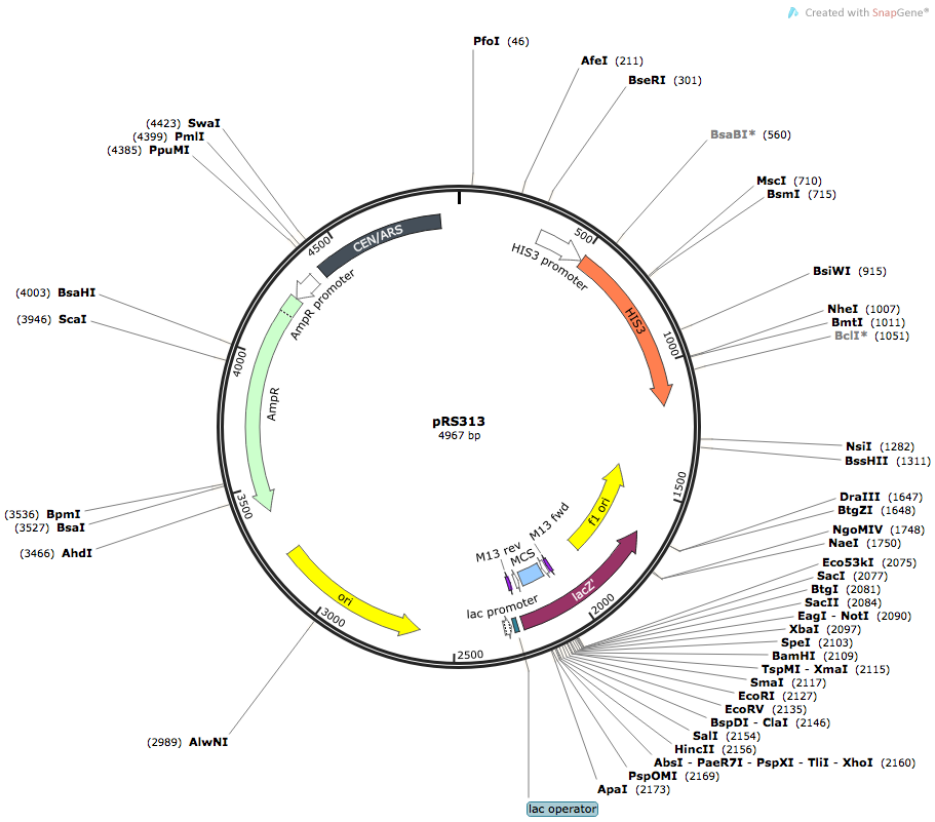
In parallel to the formaldehyde treatments to monitor the changes in growth patterns between the wildtype and SFA1 mutants, western blotting using α-SFA1 is needed to determine if there are any expression differences between the wildtype proteins and the mutants. In addition, further analysis of the role and function of the conserved cysteines can be explored using co-immunoprecipitation techniques to determine if the conserved cysteines play a role in interacting with other proteins, such as thioredoxins or glutaredoxins, *in vivo*.

APPENDIX

A. SUPPLEMENTAL DATA AND FIGURES

Supplementary Figure 1. The pRS313 plasmid

The plasmid pRS313 that AtGSNOR, AtGSNOR mutants, and SFA1 were cloned into, and introduced into the sfa1Δ cells. Plasmid pRS313 contains a *HIS3* gene that allows for selection in minus His media. It is a low copy plasmid with a centromere sequence (CEN). (Snapgene, 2017)



Supplementary Table 1. The b and y ion corresponding masses after LC-MS/MS analysis

This table reports the distribution of b and y ions and their corresponding masses after LC-MS/MS analysis. The unmodified peptide b and y ions (A) and the S-glutathionylated peptide b and y ions (B) are listed after treatment with 2 mM GSNO for 3 hr. The unmodified peptide b and y ions (C) and the S-glutathionylated b and y ions (D) are listed after treatment with 2 mM CysNO and 4 mM GSH for 3 hr. The red signifies confident b ion matches from Proteome Discoverer 2.0, while the blue signifies confident y ions.

A

#1	b^{+} (mass)	b^{2+}(mass)	b^{3+}(mass)	Seq.	y^{+}(mass)	y^{2+}(mass)	y^{3+}(mass)	#2
1	116.03423	58.52075	39.34959	D				32
2	253.09314	127.05021	85.03590	H	3435.65608	1718.33168	1145.89021	31
3	368.12009	184.56368	123.37821	D	3298.59717	1649.80222	1100.20391	30
4	496.21506	248.61117	166.07654	K	3183.57022	1592.28875	1061.86159	29
5	593.26783	297.13755	198.42746	P	3055.47525	1528.24126	1019.16327	28
6	706.35190	353.67959	236.12215	I	2958.42248	1479.71488	986.81234	27
7	834.41048	417.70888	278.80834	Q	2845.33841	1423.17284	949.11765	26
8	963.45308	482.23018	321.82254	E	2717.27983	1359.14355	906.43146	25
9	1062.52150	531.76439	354.84535	V	2588.23723	1294.62225	863.41726	24
10	1175.60557	588.30642	392.54004	I	2489.16881	1245.08804	830.39445	23
11	1274.67399	637.84063	425.56285	V	2376.08474	1188.54601	792.69976	22
12	1389.70094	695.35411	463.90516	D	2277.01632	1139.01180	759.67696	21
13	1502.78501	751.89614	501.59985	L	2161.98937	1081.49832	721.33464	20
14	1603.83269	802.41998	535.28241	T	2048.90530	1024.95629	683.63995	19
15	1718.85964	859.93346	573.62473	D	1947.85762	974.43245	649.95739	18
16	1775.88111	888.44419	592.63189	G	1832.83067	916.91897	611.61507	17
17	1832.90258	916.95493	611.63904	G	1775.80920	888.40824	592.60792	16
18	1931.97100	966.48914	644.66185	V	1718.78773	859.89750	573.60076	15
19	2046.99795	1024.00261	683.00417	D	1619.71931	810.36329	540.57795	14
20	2210.06127	1105.53427	737.35861	Y	1504.69236	752.84982	502.23564	13
21	2297.09330	1149.05029	766.36928	S	1341.62904	671.31816	447.88120	12
22	2444.16172	1222.58450	815.39209	F	1254.59701	627.80214	418.87052	11
23	2573.20432	1287.10580	858.40629	E	1107.52859	554.26793	369.84771	10
24	2676.21351	1338.61039	892.74269	C	978.48599	489.74663	326.83351	9
25	2789.29758	1395.15243	930.43738	I	875.47680	438.24204	292.49712	8
26	2846.31905	1423.66316	949.44453	G	762.39273	381.70000	254.80243	7
27	2960.36198	1480.68463	987.45884	N	705.37126	353.18927	235.79527	6
28	3059.43040	1530.21884	1020.48165	V	591.32833	296.16780	197.78096	5
29	3146.46243	1573.73485	1049.49233	S	492.25991	246.63359	164.75815	4
30	3245.53085	1623.26906	1082.51513	V	405.22788	203.11758	135.74748	3
31	3376.57135	1688.78931	1126.19530	M	306.15946	153.58337	102.72467	2
32				R	175.11896	88.06312	59.04450	1

B

#1	b^{+}(mass)	b^{2+}(mass)	b^{3+}(mass)	Seq.	y^{+}(mass)	y^{2+}(mass)	y^{3+}(mass)	#2
1	116.03423	58.52075	39.34959	D				32
2	253.09314	127.05021	85.03590	H	3740.72424	1870.86576	1247.57960	31
3	368.12009	184.56368	123.37821	D	3603.66533	1802.33630	1201.89329	30
4	496.21506	248.61117	166.07654	K	3488.63838	1744.82283	1163.55098	29
5	593.26783	297.13755	198.42746	P	3360.54341	1680.77534	1120.85265	28
6	706.35190	353.67959	236.12215	I	3263.49064	1632.24896	1088.50173	27
7	834.41048	417.70888	278.80834	Q	3150.40657	1575.70692	1050.80704	26
8	963.45308	482.23018	321.82254	E	3022.34799	1511.67763	1008.12085	25
9	1062.52150	531.76439	354.84535	V	2893.30539	1447.15633	965.10665	24
10	1175.60557	588.30642	392.54004	I	2794.23697	1397.62212	932.08384	23
11	1274.67399	637.84063	425.56285	V	2681.15290	1341.08009	894.38915	22
12	1389.70094	695.35411	463.90516	D	2582.08448	1291.54588	861.36634	21
13	1502.78501	751.89614	501.59985	L	2467.05753	1234.03240	823.02403	20
14	1603.83269	802.41998	535.28241	T	2353.97346	1177.49037	785.32934	19
15	1718.85964	859.93346	573.62473	D	2252.92578	1126.96653	751.64678	18
16	1775.88111	888.44419	592.63189	G	2137.89883	1069.45305	713.30446	17
17	1832.90258	916.95493	611.63904	G	2080.87736	1040.94232	694.29730	16
18	1931.97100	966.48914	644.66185	V	2023.85589	1012.43158	675.29015	15
19	2046.99795	1024.00261	683.00417	D	1924.78747	962.89737	642.26734	14
20	2210.06127	1105.53427	737.35861	Y	1809.76052	905.38390	603.92502	13
21	2297.09330	1149.05029	766.36928	S	1646.69720	823.85224	549.57058	12
22	2444.16172	1222.58450	815.39209	F	1559.66517	780.33622	520.55991	11
23	2573.20432	1287.10580	858.40629	E	1412.59675	706.80201	471.53710	10
24	2981.28166	1491.14447	994.43207	C-Glutathione	1283.55415	642.28071	428.52290	9
25	3094.36573	1547.68650	1032.12676	I	875.47680	438.24204	292.49712	8
26	3151.38720	1576.19724	1051.13392	G	762.39273	381.70000	254.80243	7
27	3265.43013	1633.21870	1089.14823	N	705.37126	353.18927	235.79527	6
28	3364.49855	1682.75291	1122.17104	V	591.32833	296.16780	197.78096	5
29	3451.53058	1726.26893	1151.18171	S	492.25991	246.63359	164.75815	4
30	3550.59900	1775.80314	1184.20452	V	405.22788	203.11758	135.74748	3
31	3681.63950	1841.32339	1227.88469	M	306.15946	153.58337	102.72467	2
32				R	175.11896	88.06312	59.04450	1

C

#1	b^{+}(mass)	b^{2+}(mass)	b^{3+}(mass)	Seq.	y^{+}(mass)	y^{2+}(mass)	y^{3+}(mass)	#2
1	116.03423	58.52075	39.34959	D				32
2	253.09314	127.05021	85.03590	H	3435.65608	1718.33168	1145.89021	31
3	368.12009	184.56368	123.37821	D	3298.59717	1649.80222	1100.20391	30
4	496.21506	248.61117	166.07654	K	3183.57022	1592.28875	1061.86159	29
5	593.26783	297.13755	198.42746	P	3055.47525	1528.24126	1019.16327	28
6	706.35190	353.67959	236.12215	I	2958.42248	1479.71488	986.81234	27
7	834.41048	417.70888	278.80834	Q	2845.33841	1423.17284	949.11765	26
8	963.45308	482.23018	321.82254	E	2717.27983	1359.14355	906.43146	25
9	1062.52150	531.76439	354.84535	V	2588.23723	1294.62225	863.41726	24
10	1175.60557	588.30642	392.54004	I	2489.16881	1245.08804	830.39445	23
11	1274.67399	637.84063	425.56285	V	2376.08474	1188.54601	792.69976	22
12	1389.70094	695.35411	463.90516	D	2277.01632	1139.01180	759.67696	21
13	1502.78501	751.89614	501.59985	L	2161.98937	1081.49832	721.33464	20
14	1603.83269	802.41998	535.28241	T	2048.90530	1024.95629	683.63995	19
15	1718.85964	859.93346	573.62473	D	1947.85762	974.43245	649.95739	18
16	1775.88111	888.44419	592.63189	G	1832.83067	916.91897	611.61507	17
17	1832.90258	916.95493	611.63904	G	1775.80920	888.40824	592.60792	16
18	1931.97100	966.48914	644.66185	V	1718.78773	859.89750	573.60076	15
19	2046.99795	1024.00261	683.00417	D	1619.71931	810.36329	540.57795	14
20	2210.06127	1105.53427	737.35861	Y	1504.69236	752.84982	502.23564	13
21	2297.09330	1149.05029	766.36928	S	1341.62904	671.31816	447.88120	12
22	2444.16172	1222.58450	815.39209	F	1254.59701	627.80214	418.87052	11
23	2573.20432	1287.10580	858.40629	E	1107.52859	554.26793	369.84771	10
24	2676.21351	1338.61039	892.74269	C	978.48599	489.74663	326.83351	9
25	2789.29758	1395.15243	930.43738	I	875.47680	438.24204	292.49712	8
26	2846.31905	1423.66316	949.44453	G	762.39273	381.70000	254.80243	7
27	2960.36198	1480.68463	987.45884	N	705.37126	353.18927	235.79527	6
28	3059.43040	1530.21884	1020.48165	V	591.32833	296.16780	197.78096	5
29	3146.46243	1573.73485	1049.49233	S	492.25991	246.63359	164.75815	4
30	3245.53085	1623.26906	1082.51513	V	405.22788	203.11758	135.74748	3
31	3376.57135	1688.78931	1126.19530	M	306.15946	153.58337	102.72467	2
32				R	175.11896	88.06312	59.04450	1

D

#1	b^{+}(mass)	b^{2+}(mass)	b^{3+}(mass)	Seq.	y^{+}(mass)	y^{2+}(mass)	y^{3+}(mass)	#2
1	116.03423	58.52075	39.34959	D				32
2	253.09314	127.05021	85.03590	H	3740.72424	1870.86576	1247.57960	31
3	368.12009	184.56368	123.37821	D	3603.66533	1802.33630	1201.89329	30
4	496.21506	248.61117	166.07654	K	3488.63838	1744.82283	1163.55098	29
5	593.26783	297.13755	198.42746	P	3360.54341	1680.77534	1120.85265	28
6	706.35190	353.67959	236.12215	I	3263.49064	1632.24896	1088.50173	27
7	834.41048	417.70888	278.80834	Q	3150.40657	1575.70692	1050.80704	26
8	963.45308	482.23018	321.82254	E	3022.34799	1511.67763	1008.12085	25
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10	1175.60557	588.30642	392.54004	I	2794.23697	1397.62212	932.08384	23
11	1274.67399	637.84063	425.56285	V	2681.15290	1341.08009	894.38915	22
12	1389.70094	695.35411	463.90516	D	2582.08448	1291.54588	861.36634	21
13	1502.78501	751.89614	501.59985	L	2467.05753	1234.03240	823.02403	20
14	1603.83269	802.41998	535.28241	T	2353.97346	1177.49037	785.32934	19
15	1718.85964	859.93346	573.62473	D	2252.92578	1126.96653	751.64678	18
16	1775.88111	888.44419	592.63189	G	2137.89883	1069.45305	713.30446	17
17	1832.90258	916.95493	611.63904	G	2080.87736	1040.94232	694.29730	16
18	1931.97100	966.48914	644.66185	V	2023.85589	1012.43158	675.29015	15
19	2046.99795	1024.00261	683.00417	D	1924.78747	962.89737	642.26734	14
20	2210.06127	1105.53427	737.35861	Y	1809.76052	905.38390	603.92502	13
21	2297.09330	1149.05029	766.36928	S	1646.69720	823.85224	549.57058	12
22	2444.16172	1222.58450	815.39209	F	1559.66517	780.33622	520.55991	11
23	2573.20432	1287.10580	858.40629	E	1412.59675	706.80201	471.53710	10
24	2981.28166	1491.14447	994.43207	C-Glutathione	1283.55415	642.28071	428.52290	9
25	3094.36573	1547.68650	1032.12676	I	875.47680	438.24204	292.49712	8
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31	3681.63950	1841.32339	1227.88469	M	306.15946	153.58337	102.72467	2
32				R	175.11896	88.06312	59.04450	1

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